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VENOM HEMOLYSIS AFTER SPLENECTOMY, INCLUDING THE RESISTANCE OF THE ERYTHROCYTES OF NORMAL DOGS TO THE HEMOLYTIC ACTIVITY OF COBRA VENOM.

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Several investigators have shown that the resistance of erythrocytes to various hemolytic agents is increased after splenectomy. The earlier investigations by Pugliese and Luzzatti (1), Vast (2), Joannovics (3), and Banti (4) were conducted by injecting such hemolytic agents as pyrodin and toluylendiamine into animals before and after splenectomy, and noting in a general manner the increased resistance to hemolysis *in vivo* following the removal of the spleen.

Later investigations by Chalier and Charlet (5), and more particularly by Pearce and his associates (6), have shown the increased resistance of the blood cells to hemolytic agents after splenectomy by experiments *in vivo* and *in vitro*. Chalier and Charlet (5) first drew attention to a slight increase in the resistance of dog erythrocytes to hypotonic salt solutions 10 to 12 days after splenectomy. In a series of researches by Pearce and his associates, Karsner, Austin, Peet, Krumbhaar, and Musser (6, 7, 8, 9), it was shown that the erythrocytes of dogs after splenectomy acquire an increased resistance to hypotonic salt solutions as determined by experiments *in vitro*, and to the influence of specific anti-dog hemolytic serum as shown by experiments *in vivo* and *in vitro*. These investigations have also shown that the increased resistance to hemolysis is due to changes in the erythrocytes themselves.

The relation of splenectomy to the resistance of erythrocytes to the hemolytic activity of cobra venom has not heretofore been investigated. Since the phenomenon of venom hemolysis appears to be intimately associated with the lecithin or ether-soluble activators present in erythrocytes, particularly fatty acids and their soluble soaps, and since the removal of the spleen has been shown to alter the content of fatty substances in the blood, it would appear that an investigation concerning the resistance of erythrocytes to venom before and after splenectomy may yield additional information in this problem.

While the exact nature of venom hemolysis is as yet unknown, the researches in this field indicate that the substance or substances within the erythrocytes acting with the hemolysin in the venom is of a lipoidal nature. Flexner and Noguchi (10), who originally showed that cobra venom is the most active hemolytically of a number of venoms studied, and that the erythrocytes of the dog are susceptible to its influence, believed that the venom hemolysin was activated by serum complement. Later researches by Kyes (11, 12) showed that venom causes the hemolysis of erythrocytes in the absence of serum, and Kyes and Sachs (13) believed that the activating principle was a lecithin contained in the erythrocytes, for which they proposed the name "endocomplement." Noguchi (14), on the other hand, claims that the fatty acids, neutral fats, and soluble soaps in the serum and erythrocytes are the active agents; while other investigators have attempted to correlate these opposing views on the basis that the fatty acids and soaps act as indirect activating agents in venom hemolysis, in that they possess the power of modifying the cell and rendering the intracellular lecithin available for the activation of the venom hemolysin.

The investigations of King (15) indicate that the spleen is concerned in regulating the quantity of unsaturated fatty acids, which are active hemolytic agents, in the blood, and that the benefits derived by splenectomy in pernicious anemia and other diseases, as described by Eppinger (16) and others, is to be ascribed to a reduction of the quantity of these agents in the blood. King has found in experiments conducted with dogs, that the removal of the spleen is followed by a reduction of the unsaturated fatty acids in the serum with an increase of total fats and cholesterol, and that the presence of both the latter and especially of cholesterol, which is well known to possess antihemolytic properties, may account for the increased resistance of the blood to hemolytic agents after splenectomy. However, as shown by Pearce and his associates, the resistance of the blood to hemolytic agents after splenectomy is due to changes in the erythrocytes themselves, which is not explainable on the basis of King's results, especially since King found that the quantity of antihemolytic cholesterol in the corpuscles was decreased after splenectomy, which, it would appear, should render the cells more susceptible to hemolysis, instead of the reverse. Recently Dubin and Pearce (17) reported that analysis, before and after splenectomy, of the blood of dogs shows practically no change in the amount of total fats and unsaturated fatty acids, as expressed by the iodine value.

In the investigation by Kolmer and Pearce (18), on the influence of splenectomy upon the phenomenon of non-specific complement fixation sometimes shown by normal rabbit and dog serum, which has been found by Kolmer (19) to be due largely to lipoid substances in the serum, the removal of the spleen did not materially alter the non-specific complement-binding power of the serum, whereas lipoid-solving anesthetics, such as chloroform and ether, temporarily removed this property of dog and rabbit sera.

The results of my studies herein reported show that the erythrocytes acquire a temporary increased resistance to venom hemolysis after splenectomy; whether or not this increased resistance is due to a decrease of the lipoidal activator within the cells consequent to the removal of the spleen has not been investigated, but the investigations mentioned show that the phenomenon of venom hemolysis is intimately associated with lipoids and that the spleen may exert an influence upon the lipoidal substances in both corpuscles and serum. Further researches along these lines may explain in definite terms the nature of the increased resistance of erythrocytes after splenectomy, which, at present, is not understood.

Method of Study.

Two separate series of dogs were used in the present investigation; in Series A four animals were splenectomized and two used as controls; in Series B three were splenectomized and two used as controls.

Two or three preliminary tests of the resistance of the washed erythrocytes of each dog to cobra venom were made before the splenectomies were performed, in order to obtain a venom index for each dog. In addition, a large number of normal dogs were examined in order to gain more definite information regarding the normal resistance of the erythrocytes of the dogs with the technique employed.

All the animals were kept on a mixed diet of table scraps, consisting of meat, bread, cereals, and vegetables. As shown by Pearce, Austin, and Pepper (20), the anemia following splenectomy (21) is most marked on this cooked diet as contrasted with a diet of raw meat.

Venom Tests.—One lot of cobra venom was used throughout the study.[1] The dried venom was kept tightly stoppered at room temperature and a stock 1:1,000 solution in normal salt solution (0.85 per cent) was freshly prepared each time the venom tests were conducted. From this stock dilution further dilutions with normal salt solution ranging from 1:5,000 to 1:70,000 were prepared.

Twelve dilutions of venom in amounts of 1 cc. each were used

[1] The venom was kindly furnished by Dr. Hideyo Noguchi.

routinely with each blood. To each tube was added 1 cc. of a 4 per cent suspension of washed cells; this doubled the dilutions so that they now ranged from 1:10,000 to 1:140,000. After gentle mixing, the test-tubes were incubated at 37°C. for 2 hours and placed in a refrigerator over night when the readings were made.

In each experiment a hemolytic scale was prepared by dissolving 1 cc. of the mixed cells of four to six dogs in 49 cc. of distilled water. From this 100 per cent solution of 2 per cent of cells further dilutions of hemoglobin were prepared ranging from 5 to 100 per cent. With these the degree of hemolysis in the venom tests were read off and recorded according to the amount of hemoglobin in the supernatant fluid.

Each dog was bled from an external jugular vein through a dry needle in the proportion of 4 cc. of blood in 16 cc. of a 2 per cent solution of sodium citrate in normal salt solution. These suspensions of cells were set aside in the refrigerator over night and washed three times with normal salt solution by low speed centrifugalization. After the last washing a 4 per cent suspension of each lot of cells was prepared in normal salt solution.

Every effort was made to insure uniformity in preparing the dilutions of venom and the suspensions of cells. As venom in dilution tends to deteriorate, it was considered better to prepare a fresh stock dilution for each experiment.

In Series B parallel tests with hypotonic salt solutions were conducted after the method used by Karsner and Pearce (7), except that I used 0.1 cc. of the sediment of washed cells to 3 cc. of each hypotonic salt solution, whereas they used 0.1 cc. of the corpuscular mass secured by centrifuging the blood after defibrination by gentle whipping.

RESULTS.

Venom Hemolysis of the Erythrocytes of Normal Dogs.—The results of venom tests with the blood of normal dogs and the degree of maximal and minimal resistance are shown in Table I.

As shown in Table I, the resistance of dog erythrocytes to venom hemolysin varies, and the blood of the same dog may vary to a slight extent at different periods. Apparently there is a relation

TABLE I.

Venom Hemolysis of the Washed Erythrocytes of Normal Dogs (1 Cc. of a Four Per Cent Suspension of Cells + 1 Cc. of Venom Solution).

No.	Dilution of venom and degree of hemolysis.											
	1: 10,000	1: 16,000	1: 20,000	1: 32,000	1: 40,000	1: 50,000	1: 60,000	1: 70,000	1: 80,000	1: 100,000	1: 120,000	1: 140,000
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	100	100	100	55	70	50	5	—	—	—	—	—
2	100	100	100	30	60	45	10	8	8	—	—	—
3	100	100	100	60	50	40	18	20	20	15	—	—
4	100	100	100	60	50	45	10	5	5	—	—	—
5	100	100	100	95	90	80	20	10	10	5	—	—
6	100	100	100	80	25	10	5	5	—	—	—	—
7	100	100	100	90	15	10	—	—	—	—	—	—
8	100	100	100	100	100	90	10	10	10	—	—	—
9	100	100	50	5	—	—	—	—	—	—	—	—
10	100	100	100	100	90	60	25	25	25	8	—	—
11	100	100	100	100	100	100	40	40	40	5	—	—
12	100	100	100	90	15	10	—	—	—	—	—	—
13	100	100	100	100	100	100	80	80	20	10	—	—
14	100	100	100	90	20	15	—	—	—	—	—	—
15	100	100	100	90	90	20	—	—	—	—	—	—
16	100	100	100	60	60	60	30	30	—	—	—	—
17	100	100	100	20	—	—	—	—	—	—	—	—
18	100	100	100	20	10	5	—	—	—	—	—	—
19	100	100	100	90	20	15	—	—	—	—	—	—
20	100	100	100	100	90	40	—	—	—	—	—	—
21	100	100	100	80	30	—	—	—	—	—	—	—
22	100	100	100	100	50	5	—	—	—	—	—	—
23	100	100	100	100	30	5	—	—	—	—	—	—
24	100	100	100	100	90	40	—	—	—	—	—	—
25	100	100	100	100	80	60	30	20	—	—	—	—
26	100	100	100	100	100	100	80	60	50	10	—	—
27	100	100	100	90	70	30	10	5	—	—	—	—
28	100	100	100	100	90	60	30	10	—	—	—	—
29	100	100	100	100	100	90	80	40	10	—	—	—

between the degree of anemia which an animal may have due to intestinal parasitism, distemper, or other causes, and the resistance to venom, as the cells become more susceptible to venom in the presence of an anemia. This is important in interpreting the results

of resistance tests after splenectomy on account of the temporary anemia following the removal of the spleen.

On the basis of the examination of the dogs listed in Table I, the maximal resistance of washed dog erythrocytes to cobra venom in our tests may be stated as follows:

Dilution of venom 1: 10,000 gave complete hemolysis in 100 per cent.
" " " 1: 16,000 " " " " 100 " "
" " " 1: 20,000 " " " " 97 " "
" " " 1: 32,000 " " " " 41 " "
" " " 1: 40,000 " " " " 17 " "
" " " 1: 50,000 " " " " 10 " "
" " " 1: 60,000 does not produce complete hemolysis.

In general terms, the erythrocytes of the majority of the dogs were not able to withstand an exposure to venom lower than 1: 20,000 before complete hemolysis resulted; hemolysis may begin in dilutions as high as 1: 100,000 of venom, and the point of minimal resistance is more variable, or at least more difficult to read than the point of maximal resistance.

Venom Hemolysis of the Erythrocytes of Splenectomized Dogs.— In Tables II and III are shown the results of venom tests with the blood of two of the six dogs belonging to Series A; Text-figs. 1 to 6 were plotted according to the point of maximal resistance, or the

TABLE II.

Venom Hemolysis of the Erythrocytes of Dog 4 before and after Splenectomy.

Date.	History.	Dilution of venom and degree of hemolysis.										
		1: 10,000	1: 16,000	1: 20,000	1: 32,000	1: 40,000	1: 50,000	1: 60,000	1: 70,000	1: 80,000	1: 100,000	1: 120,000	1: 140,000
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Oct. 19	1st preliminary.....	100	100	100	90	90	40	5	—	—	—	—	—
" 29	2nd "	100	100	100	60	50	45	10	5	5	—	—	—
Nov. 4	3rd "	100	100	100	50	40	20	10	—	—	—	—	—
" 10	5 days after splenectomy...	100	90	25	—	—	—	—	—	—	—	—	—
" 17	12 " " "	100	50	30	5	—	—	—	—	—	—	—	—
Dec. 7	32 " " "	100	100	100	100	90	20	10	5	—	—	—	—
" 15	40 " " "	100	100	100	100	90	80	80	60	—	—	—	—

TABLE III.

Venom Hemolysis of the Erythrocytes of Dog 2 Used as a Control.

Date.	History.	Dilution of venom and degree of hemolysis.											
		1:10,000	1:16,000	1:20,000	1:32,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	1:120,000	1:140,000
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Oct. 19	1st examination.	100	100	100	100	90	80	60	20	10	—	—	—
" 29	2nd "	100	100	100	30	60	45	10	8	8	—	—	—
Nov. 4	3rd "	100	100	100	90	90	80	60	30	20	10	—	—
" 10	4th "	100	100	100	80	70	60	30	20	20	—	—	—
" 17	5th "	100	100	100	60	40	35	10	—	—	—	—	—
Dec. 7	6th "	100	100	100	100	80	40	10	5	—	—	—	—
" 15	7th "	100	100	100	100	60	50	5	—	—	—	—	—

highest dilution of venom causing complete hemolysis in the various tests. In this series venom tests were conducted 5, 12, 32, and 40 days after splenectomy.

In Series B venom tests were conducted at intervals varying from 4 days to 4 months after splenectomy; in Table IV are shown

TABLE IV.

Venom Hemolysis of the Erythrocytes of Dog 25 before and after Splenectomy.

Date.	History.	Dilution of venom and degree of hemolysis.											
		1:10,000	1:16,000	1:20,000	1:32,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	1:120,000	1:140,000
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Feb. 1	1st preliminary.	100	100	100	100	80	60	30	10	—	—	—	—
" 8	2nd "	100	100	100	90	40	20	—	—	—	—	—	—
" 14	4 days after splenectomy.	100	90	30	10	—	—	—	—	—	—	—	—
" 23	13 " " "	100	95	80	60	30	10	—	—	—	—	—	—
" 29	19 " " "	100	100	90	80	40	5	—	—	—	—	—	—
Mar. 14	33 " " "	100	100	100	90	40	10	—	—	—	—	—	—
" 22	6 wks. " "	100	100	100	100	90	30	10	5	—	—	—	—
Apr. 20	10 " " "	100	100	100	100	100	80	40	10	—	—	—	—
May 25	4 mos. " "	100	100	100	100	100	80	60	10	—	—	—	—

the results observed with one dog of this series, and a summary of the results is shown in Text-figs. 7 to 11, according to the point of maximal resistance, or the highest dilution of venom in each test causing complete hemolysis of the cells.

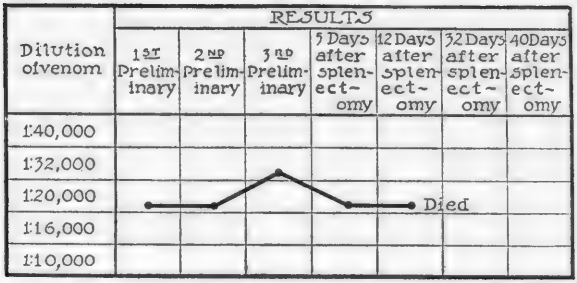
At the time of the venom tests parallel tests with hypotonic salt solutions were conducted for the purpose of a comparative study. Solutions of sodium chloride ranging from 0.3 to 0.575 per cent were used in each experiment; Table V shows the results observed with one dog of this series. In Text-figs. 7 to 11 I have included a summary of the experiments; the percentage of salt solution given being the point of maximal resistance, or the highest percentage of salt causing just complete hemolysis.

TABLE V.

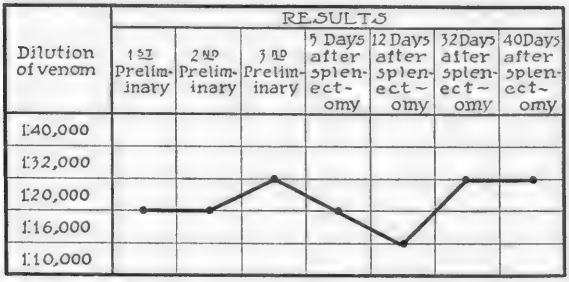
Resistance of the Erythrocytes of Dog 25 before and after Splenectomy, to Hypotonic Salt Solutions.

Date.	History.	Salt solution and degree of hemolysis.											
		0.3	0.325	0.35	0.375	0.4	0.425	0.45	0.475	0.5	0.525	0.55	0.575
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Feb. 1	1st preliminary.	100	100	100	100	90	80	40	30	10	5	—	—
" 8	2nd "	100	100	100	100	100	90	40	30	20	10	—	—
" 14	4 days after splenectomy.	100	100	100	90	90	80	50	40	10	5	—	—
" 23	13 " " "	100	100	100	90	60	50	30	5	—	—	—	—
" 29	19 " " "	100	100	80	30	10	10	5	5	—	—	—	—
Mar. 14	33 " " "	100	100	90	80	60	30	20	5	—	—	—	—
" 22	6 wks. " "	100	100	90	80	80	60	30	20	5	—	—	—
Apr. 20	10 " " "	100	100	100	90	80	60	60	20	10	5	—	—
May 25	4 mos. " "	100	100	100	90	90	80	40	20	10	—	—	—

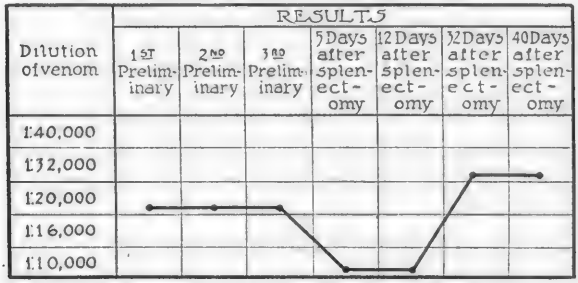
The results of my experiments with the two series of dogs may be summarized as follows: (1) The resistance of the erythrocytes of normal dogs to the hemolytic action of cobra venom is likely to vary to a slight degree in different tests, as shown in the preliminary tests with Dog 4 in Series A, and Dogs 25, 26, 28, and 29 in Series B. While every effort was made to insure a uniform technique in successive experiments, it is possible that these variations were due in part at least to variations in the strength of venom or injury to the



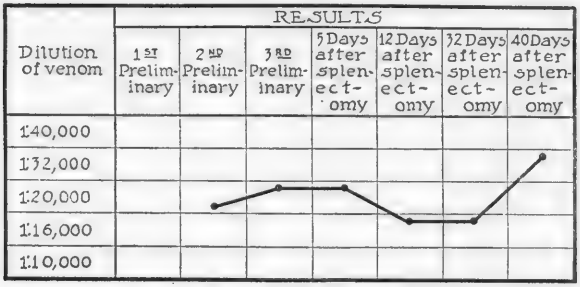
TEXT-FIG. 1. Maximal resistance of the erythrocytes of Dog 1 before and after splenectomy.



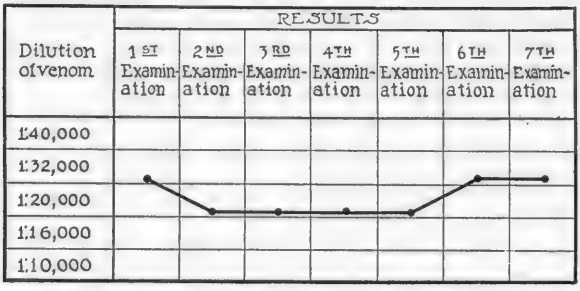
TEXT-FIG. 2. Maximal resistance of the erythrocytes of Dog 3 before and after splenectomy.



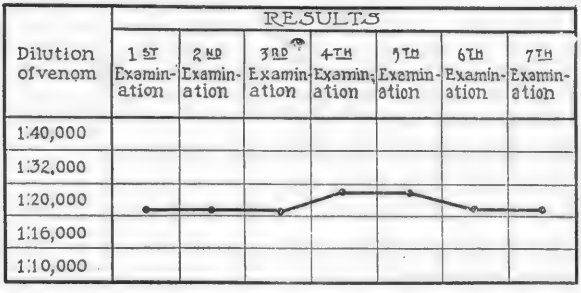
TEXT-FIG. 3. Maximal resistance of the erythrocytes of Dog 4 before and after splenectomy.



TEXT-FIG. 4. Maximal resistance of the erythrocytes of Dog 5 before and after splenectomy.

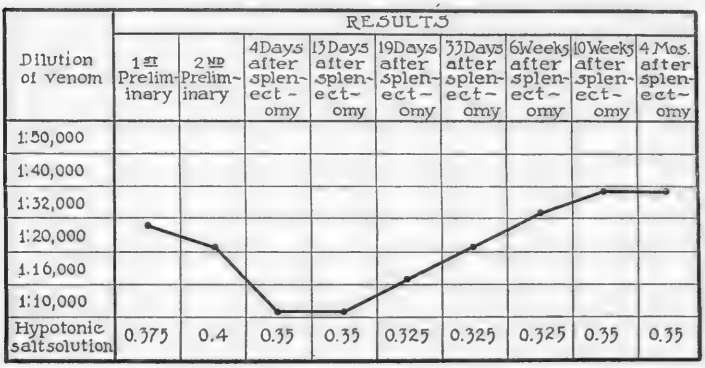


TEXT-FIG. 5. Maximal resistance of the erythrocytes of Dog 2 used as a control.

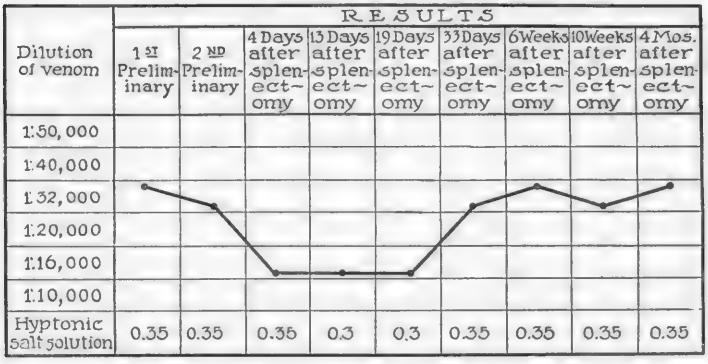


TEXT-FIG. 6. Maximal resistance of the erythrocytes of Dog 6 used as a control.

cells during the washing. At all events it would appear necessary to bear in mind these possible variations in interpreting the influence of the removal of the spleen upon the resistance of the erythrocytes.



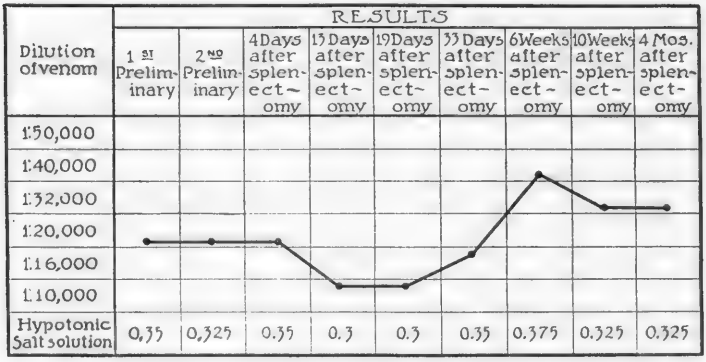
TEXT-FIG. 7. Maximal resistance of the erythrocytes of Dog 25 before and after splenectomy.



TEXT-FIG. 8. Maximal resistance of the erythrocytes of Dog 26 before and after splenectomy.

(2) On the 4th and 5th days after splenectomy the resistance of the erythrocytes to cobra venom was increased to a well marked extent in the majority of the dogs. On the 12th and 13th days after splenectomy

this increased resistance was still well marked and persisted in most instances for a period of 19 days or 3 weeks, when the erythrocytes gradually became more and more vulnerable to the influence of venom. 5 to 6 weeks after splenectomy the erythrocytes of all the dogs were as susceptible to venom as before the operation and in some instances to a greater extent. (3) Apparently the decrease of resistance to venom noted in the majority of the dogs about 4 or 5 weeks after splenectomy was coincident with an anemia, as shown by the following results of blood examinations of dogs in both series.



TEXT-FIG. 9. Maximal resistance of the erythrocytes of Dog 27 before and after splenectomy.

Dog 3.—32nd day after splenectomy. Hemoglobin, 16 per cent; erythrocytes, 1,072,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 2.)

Dog 4.—32nd day after splenectomy. Hemoglobin, 51 per cent; erythrocytes, 3,952,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 3.)

Dog 25.—10 weeks after splenectomy. Hemoglobin, 62 per cent; erythrocytes, 3,840,000. Maximal resistance to venom, 1:40,000 (a decrease of resistance). (Text-fig. 7.)

Dog 26.—10 weeks after splenectomy. Hemoglobin, 80 per cent; erythrocytes, 4,800,000. Maximal resistance to venom, 1:32,000 (normal resistance for this animal). (Text-fig. 8.)

Dog 27.—10 weeks after splenectomy. Hemoglobin, 24 per cent; erythrocytes, 1,800,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 9.)

On the other hand, the erythrocytes of the control or non-splenectomized animals did not show a decrease of resistance to venom according to the results of blood examinations.

Dog 6.—32nd day. Hemoglobin, 86 per cent; erythrocytes, 5,328,000. Maximal resistance to venom, 1: 20,000 (normal for this animal). (Text-fig. 6.)

Dilution of venom	RESULTS								
	1ST Examination	2ND Examination	3RD Examination	4TH Examination	5TH Examination	6TH Examination	7TH Examination	8TH Examination	9TH Examination
1:50,000									
1:40,000									
1:32,000									
1:20,000									
1:16,000									
1:10,000									
Hypotonic salt solution	0.35	0.35	0.35	0.375	0.4	0.375	0.375	0.375	0.35

TEXT-FIG. 10. Maximal resistance of the erythrocytes of Dog 28 used as a control.

Dilution of venom	RESULTS								
	1ST Examination	2ND Examination	3RD Examination	4TH Examination	5TH Examination	6TH Examination	7TH Examination	8TH Examination	9TH Examination
1:50,000									
1:40,000									
1:32,000									
1:20,000									
1:16,000									
1:10,000									
Hypotonic salt solution	0.325	0.350	0.35	0.35	0.375	0.35	0.325	0.35	0.35

TEXT-FIG. 11. Maximal resistance of the erythrocytes of Dog 29 used as a control.

Dog 2.—32nd day. Hemoglobin, 125 per cent; erythrocytes, 5,600,000. Maximal resistance to venom, 1: 32,000 (normal for this animal). (Text-fig. 5.)

Dog 28.—After 10 weeks. Hemoglobin, 82 per cent; erythrocytes, 4,200,000.

Maximal resistance to venom, 1:40,000 (a decrease for this animal, probably due to distemper from which the animal was suffering). (Text-fig. 10.)

Dog 29.—After 10 weeks. Hemoglobin, 87 per cent; erythrocytes, 5,100,000. Maximal resistance to venom, 1:32,000 (about normal for this animal). (Text-fig. 11.)

An increased resistance to hypotonic salt solutions was shown by the three splenectomized dogs belonging to Series B (Text-figs. 7, 8, and 9). This increase of resistance on the part of the erythrocytes to hypotonic salt solutions was apparent 13 days after the operation, and in two of the dogs (Nos. 25 and 27) persisted to a slight degree throughout the 4 months' period of observation. Dog 26 showed a slightly increased resistance on the 13th and 19th days after splenectomy, and after this time the resistance returned to the point found in the tests prior to splenectomy.

The increased resistance of the erythrocytes to venom and hypotonic salt solutions developed at or about the same intervals after splenectomy, but resistance to the latter appeared to persist for a longer time.

CONCLUSIONS.

1. The resistance of erythrocytes of dogs to the hemolytic activity of cobra venom is increased after splenectomy.

2. This increased resistance was observed as early as 4 days after splenectomy and usually persisted for a period of about 3 weeks, when the resistance gradually decreased to normal or slightly beyond.

3. The decrease of resistance to the hemolytic activity of venom for the erythrocytes of splenectomized dogs following the primary increase is apparently coincident with the anemia following splenectomy. An intercurrent infection, such as distemper, tends to reduce the resistance of erythrocytes to venom.

4. An increased resistance of the erythrocytes to hypotonic salt solutions was found with all the splenectomized dogs in which these tests were made. Increased resistance to hypotonic salt solutions apparently persists for a longer period than the increased resistance to cobra venom.

5. As the lysis of erythrocytes by venom is dependent upon the presence of certain lipoidal substances within the cells, and as the

spleen may exercise an influence over the lipoidal contents of corpuscles and serum, it is suggested that the increased resistance of erythrocytes to the hemolytic activity of venom after splenectomy is due to alterations in the lipoid content of the erythrocytes.

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THE EFFECTS OF SERUM TREATED WITH PARARABIN.

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INTRODUCTION.

In a previous publication[1] one of the writers, in collaboration with Gelat, showed that the intravenous injection of horse serum kept for 2 hours at 38°C. in the presence of one-fifth of its volume of a suspension of 0.5 per cent agar in physiological salt solution, and then separated from the agar by centrifugalization and filtration, produces in normal rabbits, when administered in adequate doses, the same symptoms that are observed after the intravenous injection of horse serum in a sero-anaphylactized rabbit.[2]

In these experiments it was also seen that the filtrate from the control mixture of agar and physiological salt solution sometimes produces a pronounced fall in the arterial pressure. This fall differs, however, from that caused by serum treated with agar, since in the first instance the pressure returns comparatively rapidly to its initial level, whereas in the second it remains for a long time at quite a low level.

Bordet has shown, in cooperation with one of us,[3] that it is possible to prepare from the relatively nitrogenous agar a product named pararabin, which constitutes a portion of the carbohydrates of agar and is practically free of nitrogen. Guinea pig serum kept for 2 or 3 hours at 37°C. in the presence of pararabin and then separated by centrifugalization and filtration from the sediment of the pararabin causes, on intravenous injection into a normal guinea pig, the same symptoms that are produced by serum similarly treated with agar; namely, tremors, dyspnea, micturition, and death after a few minutes. At autopsy, which was performed at once, dilatation of the lungs with hemorrhagic areas varying in size and number, persistence of the heart beat, and a marked retardation

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of the coagulation of the blood were observed. Guinea pig serum treated with pararabin also produces in normal guinea pigs the same symptoms as the intravenous injection of a heterologous serum into guinea pigs sensitized by previous subcutaneous or intraperitoneal injection of this heterologous serum.

It seemed to us important to investigate whether the identical results observed in guinea pigs after the intravenous injection of serum treated with agar or with pararabin exist also in rabbits injected with horse serum treated with agar or with pararabin.

Technique.

The suspension of pararabin was prepared in exactly the same manner as the suspension of agar in the above mentioned experiments of Zunz and Gelat.[1] 1 gm. of pararabin was dissolved by heating in 200 cc. of physiological salt solution. This solution was distributed in test-tubes in portions of 10 cc., after which the test-tubes were sterilized and sealed. Upon cooling, a soft jelly was formed, which was transformed, by means of vigorous shaking, into a homogeneous suspension of pararabin.

To 50 cc. of horse serum were added in each experiment 10 cc. of a suspension of pararabin; *i.e.*, five parts of serum to one of pararabin. In addition, we prepared the four following control mixtures:

1. 50 cc. of normal horse serum plus 10 cc. of physiological salt solution.
2. 50 cc. of physiological salt solution plus 10 cc. of a suspension of 0.5 per cent pararabin.
3. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of physiological salt solution.
4. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of a suspension of 0.5 per cent pararabin.

The five mixtures were kept in well corked Erlenmeyer flasks for 2 hours at 38°C. Each mixture was then submitted to rapid centrifugalization and then to filtration. Thus, the pararabin contained in the test mixture and in the second and fourth of the control mixtures was removed. In each series of experiments, five filtrates were obtained in this way. Each filtrate was injected into the marginal vein of the ear of a normal rabbit. The respiration was registered by Verdin's recording tambours, connecting with a Marey tambour, and the carotid pressure by Verdin's registering hemadynamometer. Franck's cannula was used. As an anticoagulant a solution of 1,000 cc. of water, 65 gm. of sodium bicarbonate, and 15 gm. of magnesium sulphate was employed. In order to estimate the coagulability of the blood, we discarded the first few cubic centimeters of carotid blood, contaminated by mixture with the anticoagulant solution. The blood was collected from the carotid directly into a porcelain vessel without touching the wound. The time necessary for the formation of the first fibrin floccules and for complete coagulation was recorded.

EXPERIMENTAL.

We performed only the two series of experiments described below. In both series intravenous injections were made in normal rabbits.

First Series of Experiments.

Experiment 1.—A rabbit weighing 2,420 gm. received two separate doses of 5 cc. of serum treated with pararabin. This dose corresponds to 2.07 cc. per kilo. (Table I.)

TABLE I.

First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	11.8	69	4	9.0	85
During.	12.0	70	5	9.2	84
½	11.7	70	6	9.25	85
1	11.4	70	7	9.4	81
2	9.6	89	8	9.5	83
3	8.8	105	9	9.8	74
Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.					
During.	10.25	74	5	7.6	91
½	10.05	75	6	7.8	103
1	10.8	74	7	7.7	107
2	10.0	76	8	7.7	97
3	8.6	92	9	8.0	91
4	7.7	90	10	8.1	87

The first injection of serum treated with pararabin produced a slight temporary rise of the blood pressure, occurring during the period of injection. This was succeeded by a fall which 3 minutes after the injection attained 3.2 cm. of mercury. Thereafter the pressure gradually rose again. The respiration showed an acceleration, the maximum of which corresponded with the minimum arterial pressure.

The second serum injection produced a rise in the arterial pressure during the first minute following the injection, which then recorded 1 cm. of mercury below the initial level. It next fell, reaching its

lowest level 5 minutes after the injection. This corresponds to 3.2 cm. of mercury, if the starting point is taken as the maximum of pressure observed after the second injection; but to 4.2 cm., if the starting point is taken to be the level of pressure registered at the beginning of the experiment. After this the pressure began to rise by degrees. The respiration showed a similar acceleration after the second injection of serum treated with pararabin to that after the first. But this time the maximum respiratory rate was attained only 7 minutes after the injection and in no way corresponded to the minimum arterial pressure. No defecation occurred after the injection of serum treated with pararabin. Blood drawn from the carotid 11 minutes after the second injection showed the first fibrin formation after 35 minutes. Coagulation was complete 15 minutes later, or 50 minutes after the withdrawal of the blood.

Experiment 2.—A rabbit weighing 2,335 gm. received 5 cc. of serum treated with pararabin, followed 11 minutes later by 10 cc. of the same serum. These doses correspond respectively to 2.14 and 4.28 cc. per kilo. (Table II.)

TABLE II.

First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.8	115	4	9.5	115
During.	10.0	113	5	9.0	115
½	9.95	115	6	8.6	104
1	9.8	119	7	8.6	83
2	9.2	109	8	8.45	72
3	9.25	120	9	8.45	72
			10	8.45	72
Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.					
During.	9.2	67	5	8.5	56
½	9.0	65	6	8.4	56
1	9.0	59	7	8.4	55
2	9.0	59	8	8.1	55
3	8.9	53	9	8.0	56
4	8.65	52	10	8.0	56

During the injection of serum treated with pararabin the arterial pressure rose slightly. It then gradually fell until 8 minutes after the injection it had reached a level 1.35 cm. of mercury below the initial level. The pressure was maintained at this level for 2 minutes. A second injection equal to double the first dose of the same serum treated with pararabin caused the pressure to rise 0.75 cm. of mercury, after which it again declined. 9 minutes after the second injection it had reached a level 1.8 cm. of mercury below the original level. The respiratory rate was comparatively rapid at the beginning of the experiment, but it began to diminish 6 minutes after the first injection. This retardation stopped at about the 8th minute following the injection. It was resumed directly after the second injection and stopped 4 minutes after the new injection. After the second injection there was an expulsion of twelve scybala. Blood drawn from the carotid 11 minutes after the second injection of serum treated with pararabin showed the first fibrin formation after 27 minutes; coagulation was complete 3 minutes later, or 30 minutes after the withdrawal of the blood.

Experiment 3.—A rabbit weighing 1,820 gm. received 10 cc. of serum treated with pararabin, corresponding to 5.49 cc. per kilo. (Table III.)

TABLE III.

Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.6	77	5	9.6	99
During.	10.9	67	6	9.5	93
½	10.7	69	7	9.6	82
1	10.3	77	8	9.6	64
2	10.1	92	9	9.7	67
3	9.6	96	10	9.7	69
4	9.6	88	11	9.8	65
			12	9.8	64

The intravenous injection of serum treated with pararabin produced in this case, after a slight temporary rise of blood pressure, a gradual fall which 6 minutes after the beginning of the injection reached a level only 1.1 cm. of mercury below the initial level. After

this the pressure rose again slowly. 2 minutes after the injection the respiratory rate began to increase, and this lasted for 5 minutes. The respiratory rate showed two maxima, the first occurring 3 minutes after the beginning of the injection, and the second 2 minutes later. There was no defecation. Unfortunately the time required for the coagulation of the blood was not recorded.

Experiment 4.—A rabbit weighing 1,915 gm. received 5 cc. of a filtrate of pararabin and physiological salt solution; 15 minutes later 5 cc. of a filtrate of serum and physiological salt solution were given. This corresponds to 2.61 cc. per kilo. (Table IV.)

TABLE IV.

Injection of Filtrate of Pararabin and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.0	44	6	8.6	44
During.	9.0	43	7	8.45	45
½	9.0	45	8	8.5	46
1	8.95	46	9	8.3	46
2	8.9	45	10	8.3	46
3	8.9	44	11	8.15	45
4	8.8	48	12	8.0	45
5	8.7	44	13	8.1	45
			14	8.0	46

Injection of Filtrate of Normal Horse Serum and Physiological Salt Solution (Control).

During.	8.55	46	3	8.15	43
½	8.5	44	4	8.1	41
1	8.45	44	5	8.05	42
2	8.15	43	6	7.95	43

The arterial pressure gradually fell after the injection of the filtrate of pararabin and physiological salt solution. This fall attained its maximum (1 cm. of mercury) 12 minutes after the injection. After the intravenous injection of a filtrate of serum and physiological salt solution, the pressure rose 0.55 cm. of mercury, then gradually fell again. 6 minutes after the second injection the pressure had reached 1.05 cm. of mercury below the initial level. At this point the experiment was unfortunately interrupted.

The respiratory rate remained unmodified during the experiment. There was an expulsion of seventeen scybala after the injection of the filtrate of pararabin and physiological salt solution. Blood drawn from the carotid 7 minutes after the second injection showed the first fibrin formation at the end of 12 minutes; coagulation was complete 6 minutes later, or 18 minutes after the withdrawal of the blood.

Experiment 5.—A rabbit weighing 1,830 gm. received 5 cc. of the filtrate of pararabin and serum heated to 56°C., and 7 minutes later 5 cc. of filtrate of physiological salt solution and serum heated to 56°C. This corresponds to 2.73 cc. per kilo. (Table V.)

TABLE V.

Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Pararabin (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.4	42	3	10.2	43
During.	10.5	46	4	10.2	44
½	10.4	44	5	10.2	52
1	10.3	43	6	10.2	49
2	10.2	44			

Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Physiological Salt Solution (Control).

During.	10.3	49	6	9.7	37
½	10.1	39	7	9.4	36
1	10.1	38	8	9.4	38
2	10.0	40	9	9.4	35
3	10.0	43	10	9.4	35
4	9.9	39	11	9.3	35
5	9.8	39	12	9.3	35

The injection of the filtrate of pararabin and serum heated to 56°C. produced no appreciable modification of the arterial pressure or of the respiratory rate. There was no defecation.

The injection of the filtrate of physiological salt solution and serum heated to 56°C. caused a depression of the arterial pressure, which 11 minutes later reached 1 cm. of mercury. The respiratory rate was slightly retarded. There was an expulsion of five scybala.

Blood drawn from the carotid 13 minutes after the second injec-

tion showed the first fibrin formation at the end of 15 minutes. Coagulation was complete 3 minutes later, or 18 minutes after the withdrawal of blood.

Table VI summarizes the results obtained in the first series of experiments.

TABLE VI.

Summary of First Series of Experiments.

Experiment No.	Liquid injected. Filtrate from mixture of.	Amount per kg. of liquid injected.	Effects of injection.				
			Blood pressure.	Respiratory rate.	Scybala.	Time of coagulation after blood withdrawal.	
						First fibrin formation.	Complete coagulation.
		cc.				*min.*	*min.*
1	Serum plus pararabin.	2.07	Temporary rise; then fall of 3.2 cm. Hg.	Acceleration.	0	—	—
	" " "	2.07	Temporary rise; then fall of 3.2 cm. Hg.	"	0	35	50
2	" " "	2.14	Temporary rise; then fall of 1.35 cm. Hg.	Retardation.	0	—	—
	" " "	4.28	Temporary rise; then fall of 1.8 cm. Hg.	"	12	27	30
3	" " "	5.49	Temporary rise; then fall of 1.1 cm. Hg.	Acceleration.	0	—	—
4	Physiological salt solution plus pararabin.	2.61	Immediate fall of 1.0 cm. Hg.	No change.	0	—	—
	Physiological salt solution plus serum.	2.61	Temporary rise; then fall of 1.05 cm. Hg.	" "	17	12	18
5	Serum heated to 56°C. plus pararabin.	2.73	No change.	" "	0	—	—
	Serum heated to 56°C. plus physiological salt solution.	2.73	Immediate fall of 1.0 cm. Hg.	Retardation.	5	15	18

From results obtained with control filtrates, it appears that the fall of pressure must exceed 1 cm. of mercury in order that it may be considered as a manifestation of anaphylactic shock. No importance should be attributed to the expulsion of scybala. But, on the other hand, every delay in the blood coagulation and every acceleration of the respiratory rate must be considered as a result of anaphylactic shock.

Thus, if we admit the above assumptions, it is seen that the injection of a filtrate of serum kept at 38°C. in the presence of pararabin produces a distinct anaphylactic shock when administered in doses of 2.07 cc. per kilo (Experiment 1), and that this effect occurs after two successive injections. No definite statement can be made about larger doses.

Second Series of Experiments.

The second series, like the first, represents results obtained with five normal rabbits.

Experiment 6.—A rabbit weighing 2,335 gm. was injected with 1 cc. of serum treated with pararabin, and 11 minutes later with 2 cc. of the same serum. These doses correspond respectively to 0.43 and 0.86 cc. per kilo. (Table VII.)

TABLE VII.

First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	11.8	53	4	10.8	46
During.	11.6	52	5	10.7	46
½	12.0	50	6	11.0	44
1	12.0	45	7	11.0	49
2	11.0	48	8	10.8	48
3	10.7	47	9	10.7	48
			10	11.2	48
Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.					
During.	11.0	48	3	10.7	46
½	10.8	48	4	10.6	45
1	11.0	48	5	10.5	45
2	11.0	46	6	10.4	45

The injection of 0.43 cc. per kilo of the serum treated with pararabin produced a fall of the arterial pressure which after 3 minutes had reached 1.1 cm. of mercury. This fall was preceded by a slight rise of pressure, which followed upon a first, very slight reduction, occurring simultaneously with the injection. The pressure then gradually rose, although a slight fall occurred during the 5th and 9th minutes. The second injection of serum treated with pararabin also showed a fall of the arterial pressure. After the experiment was discontinued, that is, 6 minutes after the injection, this fall had reached a level of 0.8 cm. of mercury.

The respiratory rate tended to diminish slightly after each of the two injections, but this phenomenon was hardly perceptible. Defecation occurred after the second injection.

Blood taken from the carotid 7 minutes after the second injection showed the first fibrin formation at the end of 18 minutes; coagulation was complete after 7 minutes more; that is, 25 minutes after the withdrawal of the blood.

Experiment 7.—A rabbit weighing 2,230 gm. received 2 cc. of serum treated with pararabin, or 0.89 cc. per kilo. (Table VIII.)

TABLE VIII.

Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	11.7	70	9	9.0	57
During.	11.0	69	10	9.0	60
½	10.7	67	11	9.1	59
1	10.3	73	12	9.15	58
2	10.0	67	13	9.25	56
3	9.7	79	14	9.4	54
4	9.4	82	15	9.3	54
5	9.2	76	16	9.3	54
6	9.2	72	17	9.4	50
7	9.2	69	18	9.3	52
8	9.0	67	19	9.3	52

The arterial pressure fell 2.7 cm. of mercury in 8 minutes. 11 minutes after the injection it rose again slowly.

A slight acceleration of the respiratory rate was noticeable 3 minutes after the injection, reaching its maximum at the end of the 4th minute. After the 9th minute the respiratory rate showed signs of diminishing. Defecation occurred after this injection.

Blood taken from the carotid 20 minutes after the injection showed the first fibrin formation after 25 minutes. Coagulation was not complete until 25 minutes later; *i.e.*, 50 minutes after the withdrawal of the blood.

Experiment 8.—A rabbit weighing 2,020 gm. received 5 cc. of serum treated with pararabin, or 2.47 cc. per kilo. (Table IX.)

TABLE IX.

Injection of Filtrate of Normal Horse Serum Treated with Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	9.4	57	9	9.4	53
During.	9.0	47	10	9.5	55
½	9.4	52	11	9.3	53
1	9.0	52	12	9.3	51
2	9.6	52	13	9.2	52
3	9.8	50	14	9.3	52
4	9.6	52	15	9.3	53
5	9.4	58	16	9.3	54
6	9.6	56	17	9.3	53
7	9.7	60	18	9.2	54
8	9.5	60	19	9.3	54
			20	9.4	54

The injection of 2.47 cc. per kilo of serum treated with pararabin failed to show any definite effects on the pressure. The pressure first of all fell 0.4 cm. of mercury, then rose 0.4 cm.; it then fell 0.4 cm. again, after which it rose 0.8 cm. of mercury. Thereafter it fluctuated between 0.2 cm. of mercury above and below the initial level.

The respiratory rate first slightly diminished, then 7 to 8 minutes after the injection rose a little above the initial figure, after which it fell slightly. No defecation took place.

Blood taken from the carotid 21 minutes after the injection showed the first fibrin formation after 30 minutes. Coagulation was not

complete until 20 minutes later; that is, 50 minutes after the withdrawal of the blood.

Experiment 9.—A rabbit weighing 1,970 gm. was injected with 2 cc. of the filtrate of pararabin and physiological salt solution; 11 minutes later it received 2 cc. of the filtrate of pararabin and serum. This corresponds to 1.02 cc. per kilo for each injection. (Table X.)

TABLE X.

Injection of Filtrate of Pararabin and Physiological Salt Solution (Control).

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.5	42	4	10.0	42
During.	10.6	43	5	9.9	35
½	10.6	44	6	10.0	39
1	10.2	42	7	10.0	38
2	10.3	42	8	10.2	40
3	10.1	39	9	10.2	45
			10	10.2	48
Injection of Filtrate of Normal Horse Serum and Pararabin.					
During.	10.3	37	4	10.3	44
½	10.0	40	5	10.5	45
1	9.8	42	6	10.4	48
2	9.9	44	7	10.4	46
3	10.2	46	8	10.2	45
			9	10.0	45

The injection of the filtrate of pararabin and physiological salt solution at first produced a slight temporary rise of the arterial pressure, which was followed after 5 minutes by a fall of 0.7 cm. of mercury. After this the pressure slowly rose again. The injection of the filtrate of pararabin and serum also produced a slight and transient rise of the pressure, which occurred during the injection. Then for the space of 1 minute the pressure fell 0.5 cm. of mercury, and rose to the initial level 4 minutes later, after which it gradually fell again. At the expiration of the experiment, which occurred 9 minutes after the second injection, a new fall of the arterial pressure had occurred, corresponding to 0.5 cm. of mercury; this fall would perhaps subsequently have continued.

The respiratory rate decreased slightly after the injection of the filtrate of pararabin and physiological salt solution. Later it returned to the initial figure and even slightly exceeded it. During the injection of the filtrate of pararabin and serum the respiratory rate diminished; later it regained and for a time even slightly exceeded the initial figure. These modifications were not very great. There was an expulsion of forty-five scybala after the second injection.

Blood taken from the carotid 10 minutes after the second injection showed the first fibrin formation at the end of 45 minutes. Coagulation was complete 10 minutes later; that is, 55 minutes after the withdrawal of the blood.

Experiment 10.—A rabbit weighing 2,140 gm. received successively injections of each of the following (Table XI):

1. 2 cc. of a filtrate of serum and pararabin.
2. 2 " " " " " " heated to 56°C. and pararabin.
3. 2 " " " " " " " " 56° " " physiological salt solution.

TABLE XI.

Injection of Filtrate of Normal Horse Serum and Pararabin.

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	*cm. Hg.*	*per min.*	*min.*	*cm. Hg.*	*per min.*
Before.	10.7	40	4	10.2	35
During.	10.9	38	5	9.4	39
½	10.6	35	6	9.2	36
1	10.6	35	7	9.0	43
2	10.4	38	8	8.4	42
3	10.0	38	9	8.8	43
Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Pararabin (Control).					
During.	9.2	42	3	9.6	42
½	9.4	42	4	9.2	43
1	9.6	40	5	9.4	45
2	9.4	39	6	9.2	44
			7	9.3	49
Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Physiological Salt Solution (Control).					
During.	9.5	45	3	9.8	48
½	9.4	45	4	10.0	49
1	9.8	47	5	9.9	50
2	9.8	48	6	9.9	48

The injection of the filtrate of serum and pararabin produced first, simultaneously with the injection, a temporary and insignificant rise of the arterial pressure, and then a fall which reached 2.3 cm. of mercury 8 minutes after the injection. But, on the other hand, the injection of the filtrates of serum heated to 56°C. and mixed either with pararabin or with physiological salt solution produced rises in the arterial pressure attaining, respectively, 0.4 cm. of mercury after 1 minute and 0.5 cm. after 4 minutes.

The respiratory rate did not suffer any appreciable modifications, although it tended to diminish slightly after the injection of the filtrate of serum and pararabin; it also showed signs of increasing after the injection of the filtrates of serum heated to 56°C. and mixed either with pararabin or physiological salt solution.

There was an expulsion of five scybala following the injection of the filtrate of pararabin and serum heated to 56°C., and an expulsion of seven scybala after the injection of the filtrate of physiological salt solution and heated serum.

Blood drawn from the carotid 7 minutes after the third injection showed the first fibrin formation after 12 minutes. Coagulation was complete 5 minutes later, or 17 minutes after the withdrawal of the blood.

Table XII shows the results of the second series of experiments.

In the second series of experiments we unfortunately omitted to note the action of the filtrate derived from the serum and physiological salt solution. But the results obtained with the three other control liquids lead us to assume that in all probability this mixture would not have caused a fall in the arterial pressure greater than 0.5 cm. of mercury after a dose of approximately 1 cc. per kilo. Such, indeed, was observed in Experiment 9, in the case of the filtrate of physiological salt solution and pararabin. The filtrates prepared with the serum heated to 56°C. caused a corresponding rise in the arterial pressure.

At all events, in Experiments 7 and 10, the serum treated with pararabin produced a distinct fall in the arterial pressure after a dose of approximately 1 cc. per kilo. Experiments 6 and 9 did not show this characteristic fall as a result of the same dose. With a dose of 2.47 cc., *i.e*, one slightly greater than that which, in the first series

TABLE XII.

Summary of Second Series of Experiments.

Experiment No.	Liquid injected. Filtrate from mixture of.	Amount per kg. of liquid injected.	Effects of injection.				
			Blood pressure.	Respiratory rate.	Scybala.	Time of coagulation after blood withdrawal.	
						First fibrin formation.	Complete coagulation.
		cc.				*min.*	*min.*
6	Serum plus pararabin.	0.43	Temporary rise; then fall of 1.1 cm. Hg.	Very slight retardation.	0	—	—
	" " "	0.86	Temporary rise; then fall of 0.8 cm. Hg.	" " "	9	18	25
7	" " "	0.89	Fall of 2.7 cm. Hg.	Slight acceleration.	5	25	50
8	" " "	2.47	Immediate modifications in both directions.	Insignificant changes in both directions.	0	30	50
9	Physiological salt solution plus pararabin.	1.02	Temporary rise; then fall of 0.7 cm. Hg.	Slight retardation.	11	—	—
	Serum plus pararabin.	1.02	Temporary rise; then fall of 0.5 cm. Hg.	" "	45	45	55
10	" " "	0.93	Temporary rise; then fall of 2.3 cm. Hg.	No change.	0	—	—
	Heated serum plus pararabin.	0.93	Rise of 0.4 cm. Hg.	" "	5	—	—
	Heated serum plus physiological salt solution.	0.93	Rise of 0.5 cm. Hg.	" "	7	12	17

of experiments, brought about a fall of pressure of 3.2 cm. of mercury and more (Experiment 1), the intravenous injection of serum treated with pararabin was seen to exert no effects on the arterial pressure.

We noted a retardation of the coagulation after each injection

of serum treated with pararabin. This phenomenon was most marked in Experiment 9, after the injection of a dose of 1.02 cc.

Intravenous injections of control filtrates (Experiment 10) sometimes caused the expulsion of several scybala. It should be stated that the only manifestation of anaphylactic shock in this connection is the expulsion of numerous scybala. This occurred in only one case out of five (Experiment 9).

The respiratory rate showed an increase, to a comparatively slight extent, in one out of five experiments (Experiment 7), which represented a dose of 0.89 cc. of serum treated with pararabin.

DISCUSSION.

If one compares the results obtained in the two series of experiments, it is seen that the quantity of filtrate derived from the serum which had remained at 38°C. in the presence of pararabin necessary to produce anaphylactic shock, varies according to the proportion of serum and of pararabin used, even when apparently identical experimental conditions are observed. This quantity is less in the second series than in the first. Each series appears to have an optimum dose. When the serum treated with pararabin is injected intravenously in quantities either greater or less than the optimum amount, it exerts a slighter action than the optimum dose, and if it varies considerably from this optimum, it may even fail to produce any anaphylactic shock whatever in normal rabbits

On the other hand, if in a similar series of experiments the same dose of serum is treated with pararabin, the phenomena of anaphylactic shock are sometimes either completely or nearly completely absent (as in Experiment 6), whereas at other times they can be distinctly observed (Experiment 7). This is not surprising, in view of the fact that it has long been known that in all investigations regarding anaphylaxis there exist great differences between individual animals subjected to the same treatment.

According to our two series of experiments, the fall in the pressure effected by the injection of the filtrate of physiological salt solution and pararabin has always been inconsiderable (0.7 cm. of mercury in Experiment 9, 1 cm. in Experiment 4), whereas the contrary has some-

ERRATUM.

On page 227, Vol. XXV, No. 2, February 1, 1917, line 20, for *2 or 3 minutes* read *20 or 30 minutes*.

On page 228, lines 28, 29, and 30 should read : *It should also be mentioned, as has been shown by the experiments of Bordet and Zunz with guinea pigs, that it is better to employ homologous serum; that is, rabbit serum.*

times been the case after the intravenous injection into a normal rabbit of a filtrate derived from a mixture of agar and physiological salt solution. In the third series of experiments described by Zunz and Gelat, this filtrate when injected in a dose of 2.22 cc. per kilo caused in 4 minutes a fall in the arterial pressure of 7.1 cm. of mercury, and represented a much greater shock even than that observed in the series of experiments with serum treated with agar. But the rate of pressure immediately increased rapidly, contrary to the phenomenon observed after the intravenous injection of serum which had remained in the presence of agar at 38°C. for 2 hours. In the three other cases observed by Zunz and Gelat, the fall of pressure was less accentuated after the intravenous injection of the filtrate of agar and physiological salt solution; *i.e.*, 1.3 cm. of mercury in 7 minutes in Experiment 4, 2.9 cm. of mercury in 8 minutes in Experiment 8, and 1.3 cm. of mercury in 6 minutes in Experiment 12.

In both series of experiments none of the other phenomena characteristic of anaphylactic shock were produced after the introduction into the blood stream of the filtrate of pararabin and physiological salt solution.

It sufficed to heat the serum to 56°C. for 2 or 3 minutes in order that the treatment with pararabin should fail to give it the power of producing the symptoms of anaphylactic shock when injected intravenously into a normal rabbit.

To sum up, serum treated with pararabin causes, as does serum treated with agar, the phenomena of anaphylactic shock when injected into normal rabbits. In both cases the fall in arterial pressure and the retardation of carotid coagulation constitute the essential characteristics of shock. But, on the other hand, it is not always possible to detect acceleration of the respiratory rate and excitability of intestinal activity.

As in the experiments on the guinea pig made by Bordet in conjunction with one of the writers, serum treated with pararabin appears to produce a less intense anaphylactic shock in the rabbit than serum treated with agar. But it is not certain that this is really the case. For there is no evidence that the proportion of one volume of a suspension of 0.5 per cent of pararabin to five volumes of fresh horse serum is the optimum. It is possible that a more intense anaphylactic shock

would be produced by diminishing or augmenting the proportion of pararabin.

At all events, as the filtrate derived from the control mixture of pararabin and physiological salt solution causes a much slighter fall in the arterial pressure than the filtrate of physiological salt solution and agar, it follows that pararabin and not agar must be employed in subsequent investigations.

The best proportions of serum and pararabin, respectively, and the optimum duration of contact at 38°C. should be determined first. This would necessitate numerous series of experiments. In each one it would be necessary to inject into different rabbits 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, and 2 cc. of serum treated with pararabin, as well as greater or less quantities, according to the results obtained. In order to start from well established facts and to avoid the individual factor which is so considerable in all investigations of anaphylaxis, it would be well to inject each dose into at least three rabbits. This would necessitate a large number of animals, which, unfortunately, we are unable to obtain under present conditions.

When the appropriate dose per kilo of serum treated with pararabin has been determined, the next step would be to make the necessary investigations of similar quantities of the control filtrates, at least three normal rabbits being employed for each liquid. It is also necessary that only one liquid (serum treated with pararabin or one of the control filtrates) should be injected into each animal. Only by this means can reliable results be obtained, for the experiments described above have demonstrated the necessity of these precautions.

It should also be mentioned, as has been shown by the experiments of Bordet and Zunz, that it is better to employ the homologous serum in the experiments with guinea pigs.

Although our experiments cannot be looked upon as other than purely preliminary attempts, nevertheless they confirm the results obtained by Bordet and Zunz in guinea pigs injected with serum kept at 38°C. in the presence of pararabin and subsequently separated from it.

SUMMARY.

Horse serum, kept for 2 hours at 38°C. in the presence of one-fifth its volume of a suspension of 0.5 per cent of pararabin in physiological salt solution and then freed by means of centrifugalization and filtration from the pararabin produced when injected in appropriate doses into a normal rabbit, a considerable and prolonged fall of the blood pressure, a distinct retardation in the coagulation of the carotid blood, and sometimes, in addition, acceleration of the respiratory rate and the expulsion of numerous scybala; that is, the various symptoms observed to occur after the intravenous injection of horse serum in sero-anaphylactized rabbits.

If the horse serum is first heated for 30 minutes to 56°C. and then treated with pararabin in the manner described above, the intravenous injection of this serum into a normal rabbit exerts no more action on the arterial pressure, the coagulability of the arterial blood, the respiratory rate, and the intestinal activity, than does normal horse serum when introduced into the vein of a normal rabbit.

STUDIES ON THE BLOOD PROTEINS.

II. The Albumin-Globulin Ratio in Experimental Intoxications and Infections.

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The changes which may occur in the partition of the blood protein fractions in different pathological conditions have been given little systematic study. For the most part, the recorded observations deal with the alteration in the albumin-globulin ratio in infections and in various immune states (1).

The present study was undertaken to determine whether the rise in the serum globulins reported in immunized animals occurred also during the immunity or tolerance to proteose intoxication, which develops in dogs with long-standing intestinal loops or after the injection of repeated, small doses of pure proteose. We have studied also the blood changes in animals which were being observed in connection with other experiments. In some of these animals symptoms of intoxication developed after the onset of peritonitis or the production of a pancreatitis or pleurisy,—conditions which have been shown to possess certain features in common with intestinal obstruction (2).

Our experimental observations have shown that the intoxication which develops after an acute or chronic obstruction of the intestine is in most instances associated with definite changes in the blood proteins. In a simple obstruction with toxic death and a rise in the non-coagulable nitrogen of the blood the serum globulins may rise to double their initial value within 48 hours after the production of the obstruction. And in the majority of such instances the globulins continue high until death. Similarly, animals with a chronic obstruction show a rise in the serum globulins. In these animals the globu-

lin increase takes place more slowly, but it may be of greater magnitude, and may show a tendency to return to its initial value.

Some of the most important observations have come from a study of the blood of animals with some of the complications met with in these loop experiments,—peritonitis, rupture of the loop, and overwhelming intoxication. In these experiments, the globulin content of the serum usually rises with marked rapidity and to the highest point. The blood of some of the animals may show a complete inversion of the normal albumin-globulin ratio. We have found this immediate rise in the blood globulins to be so constantly present in these complications that we believe its occurrence to be of value in the differential diagnosis of acute conditions associated with the sudden liberation of a toxic exudate. This observation is in harmony with previously recorded experiments (1), which have demonstrated that a number of inflammatory conditions with tissue destruction and pus formation are accompanied by a rise in the blood globulins. And from the present experiments we have additional evidence that an increase in the globulins may occur even when the inflammatory conditions are not the result of bacterial invasion, although it would appear that a combination of both factors,—bacteria and inflammatory irritants gives rise to greater alterations in the ratio.

Injections of small immunizing doses of toxic proteose, on the other hand, do not produce any marked alteration either in the percentage of the serum proteins or in the normal partition of the albumin and globulin fractions. The changes observed in the loop experiments may, however, result if the dosage is large and the attending intoxication profound.

From these experiments we have, furthermore, additional evidence that no parallelism exists between a rise in the blood globulins and the development of an immunity or tolerance. That such a tolerance resulted from the repeated proteose injections is clear from the fact that the animals were resistant to the injection of larger doses of this toxic substance; and yet observations upon the blood failed to disclose an increase in the globulin content. An occasional increase in the percentage of globulin did occur, but this was usually transitory and small.

Methods.

Dogs were used in all the experiments. The operations were done under surgical anesthesia and with the usual aseptic technique. A description of the experimental conditions studied has been given in previous communications (3).

Specimens of blood were obtained from the jugular vein previous to a feeding in order to get a clear serum, and analyses were made prior to or on the day of operation and at regular intervals thereafter.

All the determinations of the albumin, globulin, and non-protein fractions recorded in the columns of the tables were made by the micro-refractometric method of Robertson (4). The advantages of this method for these studies over those used heretofore have been discussed in a previous paper (1).

EXPERIMENTAL.

The Serum Proteins of Normal Dogs.

The percentage of total protein and the relative proportions of albumin and globulin present in normal dog serum have not been extensively studied by the more recent and more accurate methods. The high globulin figures obtained by Lewinski[1] (5) are not in accord with the more recent percentages obtained by the micro-refractometric method. The percentages obtained by Robertson and his coworkers (6) as well as our own are considerably lower (Table I). We have found the average total protein percentage obtained from a study of thirty-five normal dogs to be 6.1 per cent, of which the globulin fraction constituted 1.5 per cent. These figures are in accord with those recently obtained by Jewett (6) in a study of a smaller number of sera by the same method.

[1] According to Lewinski, the percentage composition of serum proteins in normal dogs is as follows: total protein, 6.03 per cent; serum albumin, 3.17 per cent; serum globulin, 2.26 per cent. From his figures it is clear that the serum globulins would constitute 42 per cent of the total proteins, which is 17 per cent higher than the percentage obtained by us.

TABLE I.

Albumin, Globulin, and Total Protein in the Blood Serum of Normal Dogs.

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.*
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	Nov. 26	6.2	4.8	1.4	76	24	1.5	3.1
2	" 3	5.6	4.1	1.5	71	26	2.3	2.7
3	" 3	5.7	3.6	2.1	63	37	2.0	1.7
4	" 6	6.3	5.1	1.2	80	20	1.7	4.0
5	" 10	5.8	4.4	1.4	76	24	2.1	3.1
6	" 22	5.9	4.2	1.7	70	30	1.7	2.3
7	" 29	6.3	5.2	1.1	83	17	1.7	4.8
8	" 29	6.2	5.0	1.2	80	20	1.7	4.0
9	" 29	6.4	5.3	1.1	82	18	1.6	4.5
10	Dec. 3	6.0	4.1	1.9	70	30	1.2	2.3
11	" 9	6.5	5.2	1.3	82	18	1.6	4.5
12	" 20	6.6	5.4	1.2	81	19	1.5	4.2
13	Jan. 25	5.9	4.0	1.9	68	32	1.8	2.1
14	" 28	6.6	5.6	1.0	85	15	2.2	5.6
15	Feb. 3	4.9	3.8	1.1	77	23	1.8	3.3
16	Mar. 13	4.7	4.0	0.7	85	15	2.4	5.6
17	Jan. 26	6.3	4.7	1.6	74	26	1.9	2.8
18	Feb. 2	5.7	3.7	2.0	65	35	1.7	1.9
19	" 9	7.0	5.1	1.9	72	28	2.5	2.6
20	" 11	6.3	5.0	1.3	79	21	1.8	3.7
21	" 16	6.0	5.4	0.6	90	10	2.0	9.0
22	Mar. 1	5.6	3.8	1.8	70	30	2.0	2.3
23	" 8	6.4	6.0	0.4	93	7	2.1	13.2
24	" 15	6.4	5.0	1.4	78	22	2.2	3.5
25	" 22	6.1	3.6	2.5	60	40	2.7	1.5
26	Dec. 21	6.8	4.9	1.9	72	28	1.7	2.6
27	" 30	6.5	4.6	1.9	70	30	1.8	2.3
28	Jan. 6	6.9	4.9	2.0	71	29	1.6	2.4
29	" 6	6.2	4.8	1.4	77	23	1.7	3.3
30	" 6	6.1	4.3	1.8	70	30	2.1	2.3
31	Mar. 20	6.8	4.0	2.8	60	40	1.6	1.5
32	Apr. 3	5.7	4.5	1.2	79	21	2.1	3.7
33	" 25	5.0	3.5	1.5	70	30	2.5	2.3
34	May 1	6.1	4.5	1.6	74	26	2.0	2.8
35	" 4	5.5	4.0	1.5	72	28	2.2	2.6
Average		6.1	4.6	1.5	75	25	1.6	3.0
Jewett (6)		6.13	4.82	1.31	79	21	2.0	

* The protein quotient is the figure obtained by dividing the percentage of albumin by the percentage of globulin.

From Table I it is apparent that normal dogs may show some variation in the percentages of total protein, serum albumin, and serum globulin. These normal fluctuations are not large, however, when compared with those observed in the experimental conditions studied. Whereas the protein quotient in normal animals averages 3.0 and in most instances does not fall below 1.5, some of the animals with intoxication show at one period or another a quotient much below 1.0. We have taken these normal variations into account in that determinations of the normal percentages were made previous to the study of the experimental condition.

Simple Obstruction for 5 Days. Toxic Death. Peritoneum Clean.

Dog 5.—Shepherd, mongrel, female; weight 36 pounds.

Nov. 10. Simple obstruction in the middle of the small intestine by means of a complete section and an inversion of the ends.

Nov. 11 and 12. Dog is vomiting, and there is a steady loss of weight.

Nov. 13. Much thin, yellow vomitus. Temperature 102.8° F. Weight 33 pounds.

Nov. 15. Dog is severely intoxicated. Ether anesthesia. Killed.

Autopsy.—Thorax, lungs, and heart normal. Viscera show slight congestion. Peritoneum dry and clean. There are a few fibrinous adhesions about the site of obstruction. Intestinal tract below obstruction is collapsed and mucosa normal. No congestion or ulceration.

TABLE II.

Dog 5. Simple Obstruction. Toxic Death.

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Nov. 10	5.8	4.4	1.4	76	24	2.1	3.1	Operation. Non-coagulable nitrogen in blood 34 mg. Urea nitrogen 15 mg.*
" 12	6.6	3.4	3.2	52	48	2.6	1.1	
" 13	6.4	3.4	3.0	53	47	2.4	1.1	Non-coagulable nitrogen in blood 57 mg. Urea nitrogen 20 mg.
" 15	10.7	4.8	5.9	45	55	2.6	0.8	Killed.

*In the tables non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

The protocol of Dog 5 gives the clinical history of an animal succumbing to this type of obstruction. Constant vomiting and an acute terminal intoxication are the chief clinical features. From Table II it is clear that the progressive intoxication has registered definite changes in the blood proteins. 48 hours after the operation, the serum globulins had already increased 50 per cent. This increase, it would appear, is partly at the expense of the albumin fraction. And in spite of the progress of the intoxication, no further rise in globulins took place until the day of death, when this fraction constituted 55 per cent of the total proteins. The terminal increase of the total protein to double its initial value is also noteworthy. This is probably to be accounted for by the extreme dehydration which occurred during the last few days as a result of vomiting. From the protocol and table, it is clear that the percentage rise in globulins showed no tendency to parallel the increase in the severity of the intoxication.

TABLE III.

Dog 6. Long Loop of Ileum. Volvulus of Loop.

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Nov. 19								Operation.
" 22	5.9	4.2	1.7	70	30	1.7	2.3	Non-coagulable nitrogen in blood 30 mg. Urea nitrogen 14 mg.
" 24	5.1	2.9	2.2	57	43	1.4	1.3	
" 26	5.2	3.4	1.8	65	35	1.3	1.9	Non-coagulable nitrogen in blood 32 mg. Urea nitrogen 15 mg.
" 29	5.9	4.4	1.5	74	26	1.2	2.8	
Dec. 4								Found dead on 15th day.

Long Loop of Ileum. Volvulus of Loop.

Dog 6.—Black and white female; weight 20¾ pounds.
Nov. 19. A long circular loop of ileum was made by end to end anastomosis.
Nov. 20. Dog is well. Temperature 101.2°F. Weight 20¼ pounds.
Nov. 22. Dog is fairly well. Weight 19½ pounds.
Nov. 24. No vomiting; eats.

Nov. 26. No vomiting. Dog very active and looks normal.

Nov. 27. Dog seems normal.

Dec. 4. Found dead in cage.

Autopsy.—Thorax negative. Spleen small and not engorged. Liver pale and not congested. Stomach, duodenum, and small intestine all pale and normal. Anastomosis in good condition. Loop is enormous and tense, dark, blackish green in color, and twisted about its pedicle. Mucosa intact, but grayish and probably necrotic. Peritoneal cavity shows slight injection, and contains 150 cc. of sticky fluid.

Long Loop of Ileum. Slowly Progressing Intoxication. Proteose Injection.

Dog 2.—Strong black and tan, bobtail, female; weight 29½ pounds.

Oct. 29. A long, circular loop of ileum was made by end to end anastomosis; about one-half of the small intestine was included.

Oct. 30. Dog is in good condition. Temperature 102.5°F. Weight 29½ pounds.

Nov. 1. There is much bile-stained vomitus below the cage. Temperature 101.6°F. Weight 28½ pounds.

Nov. 3. Dark, mucoid fluid below cage; partly vomitus and partly diarrhea. Pulse good.

Nov. 4. Dog much better; eats and looks well. No vomitus; no diarrhea. Temperature 102.3°F. Weight 27¼ pounds.

Nov. 5. Fluid and bloody mucous diarrhea persists.

Nov. 8. Dog has slight nasal discharge. No diarrhea. Temperature 102.2°F. Weight 25¾ pounds.

Nov. 9. Dog seems fairly well.

Nov. 11. Little diarrhea. No vomitus. Eats a little. Weight 24¼ pounds.

Nov. 13. Dog is in good condition. Passed solid stool.

Nov. 15. Injection of pure proteose (pancreas I), alcohol precipitate 20 cc. Dog is resistant to proteose.

Nov. 16. Dog losing ground. Weight 24 pounds. Temperature 102.4°F. Nasal discharge but no frank distemper.

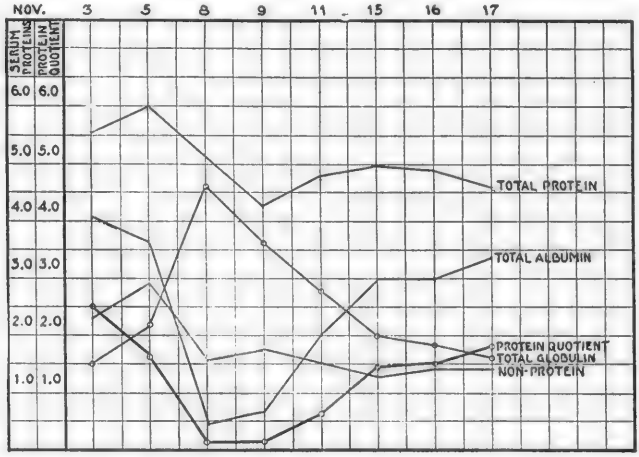
Nov. 17. Dog about the same. No vomiting. Strong and would live several days. Killed for blood.

Autopsy.—Peritoneum clean except for one small pus pocket close to the anastomosis in the loop. Peritoneal cavity contains a slight amount of turbid fluid. Stomach and small intestine are intact and pale. Anastomosis in ileum is clean and open. Liver is fatty. Bile pigments in urine and serum. The loop ends are joined, and the sutures are buried. No ulcer; mucosa has grown over the stitches. Wall of mucosa is double, but not thickened in spite of dilated loop. Mucosa everywhere pale and intact.

TABLE IV.

Dog 2. Long Loop of Ileum. Proteose Injection.

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Oct. 29								Operation.
Nov. 3	5.6	4.1	1.5	72	28	2.3	2.6	Non-coagulable nitrogen in blood 90 mg. Urea nitrogen 21 mg.
" 5	6.0	3.7	2.3	62	38	2.9	1.6	
" 8	5.1	0.5	4.6	10	90	1.5	0.11	Non-coagulable nitrogen in blood 53 mg. Urea nitrogen 20 mg.
" 9	4.3	0.65	3.6	12	88	1.7	0.13	
" 11	4.8	2.0	2.8	42	58	1.5	0.72	Non-coagulable nitrogen in blood 59 mg. Urea nitrogen 21 mg.
" 15	5.0	3.0	2.0	60	40	1.3	1.5	Non-coagulable nitrogen in blood 54 mg. Urea nitrogen 32 mg. Injection of proteose.
" 16	4.97	3.0	1.97	60	40	1.4	1.5	Non-coagulable nitrogen in blood 40 mg. Urea nitrogen 19 mg.
" 17	4.6	3.3	1.6	65	35	1.5	1.9	Killed.



TEXT-FIG. 1. Dog 2. Long loop of ileum. No peritonitis. Gradual rise in globulins.

The protocols of both of these experiments show that the intoxication in this type of closed intestinal loop is slowly progressing. Dog 6 lived 15 days, and Dog 2 was killed 19 days after the operation. The loop in these experiments was made by isolating a circular portion of the intestine and reestablishing the continuity of the rest of the intestine without obstructing the flow from the duodenum to the jejunum and ileum. The intoxication developed under these conditions is mild, and this explains the slight changes in the non-coagulable nitrogen of the blood.

Notwithstanding the mildness of the intoxication, both animals showed considerable alteration in the albumin-globulin ratio of the blood. Dog 6 is of great interest, because this animal showed no clinical symptoms of intoxication during any period of the experiment, and was found dead on the 15th day. The percentage of globulins, however, had increased 13 per cent on the 5th day (Table III), but on the day of death the globulins had returned almost to a normal level.

Dog 2 lived 3 weeks. This experiment is especially complete, since the blood examinations could be made over a longer period. In this instance, also, the toxic symptoms were not marked. The first definite rise in the globulins was noted only on the 7th day after the operation (Table IV). On the 10th day, the globulin fraction constituted 90 per cent of the total protein, which at this time showed only slight variation from its initial value. The reason for so marked a rise in the globulins of this animal is not clear. Since the total protein percentage on this day showed only little change, it must be assumed that the increase in globulins took place at the expense of the albumin fraction, which fell from an initial value of 4.1 per cent to 0.5 per cent. Following the maximum rise, the globulins began to decrease on the 13th day, gradually reaching 35 per cent on the day the animal was killed. The fluctuations in the various fractions are shown in Text-fig. 1.

Both experiments illustrate two points which deserve especial emphasis: first, the absence of any parallelism between the degree of intoxication and the percentage rise of globulins; and second, the tendency noted in several experiments for the globulin curve to return to its initial level. This would seem to indicate that the partition

of the serum protein may become normal when the cause or causes which alter the relative proportions are no longer at work.

Long Loop of Jejunum. No Peritonitis.

Dog 15.—Mongrel, black and white setter, male; weight 32¾ pounds.

Feb. 1. A high loop of jejunum was made by end to end anastomosis.

Feb. 2. Dog rather quiet. Temperature 38.9°C.

Feb. 3. Some dark brown vomitus; pulse good. Temperature 38.4°C. Weight 32 pounds.

Feb. 4. No vomiting; lively.

Feb. 8. Dog in good condition. Temperature 38.3°C. Weight 30¼ pounds.

Feb. 12. Condition the same. No vomiting.

Feb. 14. Dog is weak; pulse slow, but good volume. Temperature 38.3°C. Weight 27¾ pounds.

Feb. 16. Condition the same. Weight 26¾ pounds.

Feb. 17. Dog is losing ground. Weight 25¾ pounds.

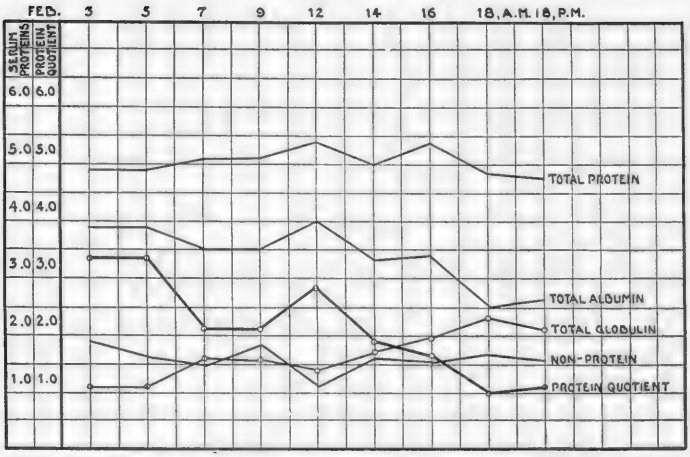
Feb. 18, 3.30 p.m. Dog is quite weak. Ether anesthesia. Killed.

Autopsy.—Thorax clean. Spleen small and fibrous. Stomach contains little fluid. Mucosa normal. Duodenum contains bright bile-stained fluid; mucosa normal. Anastomosis in good condition. Jejunum small and collapsed; mucosa normal. Peritoneal cavity moist and clean; contains a few cubic centimeters of

TABLE V.

Dog 15. Long Loop of Jejunum. No Peritonitis.

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Feb. 1								Operation.
" 3	4.9	3.8	1.1	77	23	1.8	3.3	
" 5	4.9	3.8	1.1	77	23	1.6	3.3	
" 7	5.1	3.5	1.6	68	32	1.5	2.1	Non-coagulable nitrogen in blood 29 mg.
" 9	5.1	3.5	1.6	68	32	1.7	2.1	
" 12	5.4	4.0	1.4	74	26	1.1	2.8	
" 14	5.0	3.3	1.7	66	34	1.7	1.9	
" 16	5.4	3.4	2.0	63	37	1.6	1.7	
" 18, a.m.	4.9	2.5	2.4	51	49	1.7	1.0	Non-coagulable nitrogen in blood 75 mg. Urea nitrogen 34 mg.
" 18, p.m.	4.8	2.6	2.2	54	46	1.6	1.1	Killed.



TEXT-FIG. 2. Dog 15. Long loop of jejunum. No peritonitis. Moderate rise in globulins.

blood-tinged fluid, probably due to twist of lower end of loop and omentum. Loop is huge and contains 750 cc. of dark brown syrupy fluid. Serosa normal. Mucosa intact everywhere. Few ecchymoses in washed mucosa, giving it a mottled brown and dull red color. Lower 4 inches of loop are deep red and acutely congested, due to partial twist of this end.

Long Loop of Jejunum. General Peritonitis.

Dog 4.— Mongrel, male; weight 22 pounds.

Nov. 6. Long loop of jejunum made by section and inversion of ends; jejunum joined around the loop by means of end to end anastomosis between upper and lower end.

Nov. 8. Dog looked well. Temperature 102.4°F. Weight 21 pounds.

Nov. 9. Much vomiting. Weight 20½ pounds.

Nov. 10. Dog better. No vomiting.

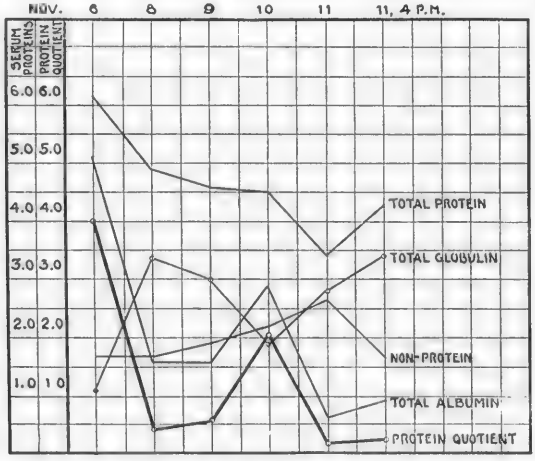
Nov. 11. Little vomiting. Temperature 100.2°F. Weight 21 pounds. 4 p.m. Dog is weak. Ether anesthesia. Killed.

Autopsy.—Thorax, heart, and lungs normal. Peritoneal cavity contains many isolated pockets of purulent exudate. A large, round worm is free in the peritoneal cavity, having escaped from the loop, which contains numerous similar worms. There are organized adhesions just below the end to end anastomosis, causing a sharp kink and probably complete obstruction. The loop contains 130 cc. of pale, slate-colored fluid with a strong odor. Mucosa of loop pale and intact. The peritonitis is probably of 1 or 2 days' duration.

TABLE VI.

Dog 4. Long Loop of Jejunum. Obstruction. General Peritonitis.

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Nov. 6	6.3	5.1	1.2	80	20	1.7	4.0	Operation.
" 8	4.9	1.6	3.3	32	68	1.7	0.47	Non-coagulable nitrogen in blood 32 mg. Urea nitrogen 13 mg.
" 9	4.6	1.6	3.0	35	65	1.9	0.54	
" 10	—	—	—	—	—	—	—	
" 11	3.4	0.6	2.8	18	82	2.6	0.2	Non-coagulable nitrogen in blood 50 mg. Urea nitrogen 22 mg.
" 11, 4 p.m.	4.3	0.9	3.4	20	80	1.6	0.2	Killed.



TEXT-FIG. 3. Dog 4. Long loop of jejunum. General peritonitis. Precipitous rise in globulins.

Tables V and VI give the changes observed in the serum proteins of animals with closed loops of the jejunum. In these dogs, also, the loops were so made that there was unobstructed flow through the duodenum. Dog 15 lived 18 days (Text-fig. 2), and showed through-

out only a mild intoxication. The percentage of globulins rose only moderately on the 6th day, and on the day the animal was killed the globulins constituted 46 per cent of the total protein. At autopsy the loop was found much distended with fluid, but the peritoneal cavity was moist and clean.

In Dog 4 the same experimental condition was produced, but this experiment illustrates one of the complications which may arise in animals with closed loops. This dog developed a peritonitis as a result of leakage from the loop, and was killed 5 days after the operation. 2 days after the loop had been isolated, the percentage of globulins had already risen from 20 to 68 per cent. The percentage of total protein fell, and, as will be seen from Text-fig. 3, the rise in globulins occurred largely at the expense of the albumin fraction.

On the day the animal was killed, the globulins constituted 82 per cent of the total protein,—a complete inversion of the normal ratio. At autopsy 130 cc. of fluid were found in the peritoneal cavity, the peritonitis appearing to be of 1 or 2 days' duration. But according to the other experiments with uncomplicated closed loops, such a rapid and marked rise in the globulin fraction is not the usual occurrence. This would indicate that the leakage of toxic material with its rapid absorption may well have commenced soon after the operation. This observation together with others furnishes good evidence that a rapid absorption of toxic exudate may give rise to an early and large increase in the percentage of serum globulin.

The Effect of Intravenous Injections of Proteose upon the Albumin-Globulin Ratio.

The immunity to proteose injections shown by dogs with long-standing closed intestinal loops indicates that the presence of a closed loop causes a chronic proteose intoxication, which gives the animal a certain degree of immunity against these toxic substances. In order to determine whether the rise in serum globulins observed in these loop experiments was associated with the development of this tolerance, the observations recorded in Table VII were made. Three normal dogs were injected intravenously with small sublethal doses of pure proteose. Injections were given at weekly intervals

TABLE VII.

Proteose Injections. Normal Albumin-Globulin Ratio.

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
7	Nov. 29	6.3	5.2	1.1	83	17	1.7	4.8	Injection of 10 cc. of cat loop proteose. Vomiting and elevation of temperature.
	Dec. 1	5.6	4.4	1.2	78	22	1.5	3.5	Dog lively and in good condition.
	" 6	6.1	4.1	2.0	67	33	1.9	2.0	Injection of 10 cc. of cat loop proteose. Dog sick; vomited.
	" 20*	5.8	4.5	1.3	77	23	1.9	3.3	Two proteose injections, one on Dec. 13 and another on Dec. 20.
8	Nov. 29	6.2	5.0	1.2	80	20	1.7	4.0	Injection of 10 cc. of peritoneal exudate proteose. Slight elevation of temperature.
	Dec. 1	5.2	3.1	2.1	59	41	1.4	1.4	
	" 6	6.6	5.2	1.4	78	22	1.7	3.5	Injection of 15 cc. of peritoneal exudate proteose. Vomiting, slight diarrhea.
	" 9	5.8	4.4	1.4	75	25	1.6	3.0	
	" 13	5.9	4.6	1.3	79	21	2.0	3.7	Injection of 15 cc. of loop fluid. Elevation of temperature; slight vomiting.
	" 20	6.0	4.4	1.6	73	27	2.0	2.7	Injection of 20 cc. of peritoneal exudate. Elevation of temperature; slight intoxication.
	Jan. 5	5.8	3.9	1.9	67	33	1.7	2.0	Injection of 25 cc. of proteose on Dec. 27 and of 35 cc. on Jan. 5. Rapid recovery. Slight intoxication.
9	Nov. 29	6.4	5.3	1.1	82	18	1.6	4.5	Injection of 6 cc. of loop proteose. Slight vomiting.
	" 29	5.9	5.1	0.8	86	14	1.4	6.1	Specimen obtained 2 hrs. after injection.
	Dec. 6	6.5	5.3	1.2	81	19	1.4	4.3	Injection of 10 cc. of loop proteose. Slight vomiting.

* The specimen was obtained immediately after the injection.

TABLE VII—*Continued.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
9	Dec. 9	6.0	4.1	1.9	68	32	2.3	2.1	
	" 13	5.6	4.2	1.4	74	26	1.8	2.8	Injection of 15 cc. of loop proteose. Elevation of temperature and vomiting.
	" 20	6.2	5.0	1.2	80	20	1.7	4.0	Injection of 20 cc. of loop proteose. Slight intoxication.
	Jan. 5	6.1	5.0	1.1	82	18	1.7	4.5	Injection of 25 cc. of proteose on Dec. 27 and 35 cc. on Jan. 5. Slight intoxication.
36	Dec. 20	6.6	5.4	1.2	81	19	1.5	4.2	Injection of 10 cc. of proteose from human peritoneal exudate; subsequent injections of 15 cc. and 25 cc. on Dec. 27 and Jan. 5. Very slight intoxication.
	Jan. 5	6.9	4.9	2.0	71	29	1.4	2.4	
37	Apr. 6	5.3	3.0	2.3	60	40	2.1	1.5	Drained loop made on Dec. 23. Dog in good condition until Apr. 6. when 27½ cc. of loop fluid proteose were injected. Vomiting, diarrhea, and intoxication.
	" 7	5.6	2.8	2.8	50	50	2.5	1.0	Died 24 hrs. later. Specimen obtained at autopsy.

and analyses of the blood were made before each injection. The proteose was prepared either from loop fluid or from peritoneal exudates. After the second injection the animals responded well to larger doses, and showed only slight signs of intoxication.

It is clear from Table VII that dogs injected with small doses of proteose do not show any alteration in the albumin-globulin ratio of the blood serum. The occasional slight rise which has been observed is within the limit of the variations noted in normal dogs. It would appear, therefore, that although the animals developed a tolerance for increasing doses of proteose, the globulin content remained unchanged. It should be emphasized that this is a slowly devel-

TABLE VIII.

The Effect of Inflammatory Conditions (Peritonitis, Pleurisy, and Pancreatitis) upon the Albumin-Globulin Ratio.

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
38	Feb. 23	7.8	3.3	4.5	42	58	2.8	0.7	*Peritonitis.* Gastrojejunostomy done on Feb. 18. Died 5 days later of general peritonitis.* Specimen obtained on day of death.
39	Apr. 21	5.3	1.0	4.3	23	77	3.9	0.3	*Pleurisy.* Injection of 1 cc. of turpentine and 5 cc. of aleuronat into right pleural cavity on Apr. 7. Second injection of 1 cc. of turpentine on Apr. 19. *Autopsy.*—Thick fibrinopurulent exudate over lungs.
16	Mar. 20	5.2	2.7	2.5	52	48	2.4	1.1	*Pleurisy.* Mar. 13. Injection of 1 cc. of turpentine and 5 cc. of 1 per cent aleuronat into right pleura. Mar. 17. Injection of ¾ cc. of turpentine and 5 cc. of aleuronat. Aspiration of 50 cc. of bloody fluid on Mar. 18 and 10 cc. on Mar. 20. *Autopsy.*—Mar. 23. Purulent and fibrinous exudate in right pleura. About 40 cc. of blood-tinged fluid obtained.
40	Apr. 21	5.6	3.0	2.6	53	47	3.2	1.1	*Pancreatitis.* Apr. 20. 10 cc. of fresh dog bile injected into pancreatic duct. *Autopsy.*—Apr. 21. Little fluid in peritoneal cavity; pancreas large, edematous; moderate hemorrhage in stroma; slight amount of fat necrosis. Not an extreme injury of gland.

* A more marked increase in the percentage of globulins associated with the development of a peritonitis was observed in Dog 4.

TABLE VIII—*Continued.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
41	Apr. 26	5.4	1.0	4.4	19	81	2.2	0.2	*Turpentine abscess and pancreatitis.* Apr. 20. Injection of 2 cc. of turpentine into left thorax. Apr. 26. Injection of 10 cc. of dog bile and glycocholic acid. Killed 7 hrs. later. *Autopsy.*—Abscesses in tissues. Pancreas large, dark purple, with extreme edema and fat necrosis.

oping tolerance for proteose which is produced under these experimental conditions.

The Effect of Peritonitis, Pancreatitis, and Pleurisy upon the Albumin-Globulin Ratio.

Table VIII contains some observations upon various inflammatory conditions in which the partition of the protein fractions was studied. In Dog 38 the peritonitis resulted from leakage following gastrojejunostomy. The high percentage of globulins noted in this experiment is in harmony with similar observations (Table VI).

Dogs with sterile pleurisy produced by the injection of turpentine and aleuronat also show a high globulin content in the serum. In these experiments the presence of bacteria in the pus formation was excluded by obtaining sterile cultures from the exudate. Notwithstanding this, however, a considerable increase in globulins occurred. This observation supplements the results of previous experiments. We have shown in a previous paper (1) that bacterial toxins obtained from bacterial growths by Berkefeld filtration may give rise to just as marked an alteration in the ratio as the injection of living and killed cultures of the organisms themselves.

The experiments with acute hemorrhagic pancreatitis produced by the injection of dog's bile into the pancreatic duct further emphasize this point. Dog 41 is of especial interest, because in this animal the pancreatitis was complicated by a turpentine abscess. This animal showed a complete inversion of the albumin-globulin ratio, the globulins constituting 81 per cent of the total protein.

TABLE IX.

Serum Proteins in Dogs with Distemper.

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
42	Nov. 8	7.0	5.6	1.4	80	20	1.4	4.0	Sept. 22. Operation, drained loop of duodenum and jejunum. Animal in good condition until Oct. 21. Developed distemper, which was quite severe on Nov. 8. *Autopsy.*—Large patches of bronchopneumonia.
43	Oct. 30	5.9	3.0	2.9	51	49	1.9	1.0	Dog in poor condition for a week before examination of blood. Profuse nasal discharge. *Autopsy.*—Lungs show only a few hemorrhagic specks.
44	Nov. 4	5.9	4.3	1.6	73	27	1.7	2.7	Slight grade of distemper.
45	Dec. 23	6.8	4.1	2.7	60	40	2.3	1.5	Acute infection. Distemper. *Autopsy.*—Hemorrhagic specks and patches in lungs.
46	Jan. 5	6.7	4.3	2.4	64	36	1.6	1.7	Died on Jan. 17 of distemper.
47*	Mar. 22	4.2	2.4	1.8	57	43	2.0	1.3	*Autopsy.*—Patches in both lungs.

* Pup.

During the course of the experiments a few other conditions were studied which deserve brief mention. An opportunity was afforded to study a few animals with distemper (Table IX). With the infection of the respiratory passages these animals may at times show

well marked signs of intoxication. Of six dogs studied only two showed any considerable alteration in the ratio. Two dogs showed a moderate change, and in two animals the albumin and globulin percentages were normal. From these few observations no definite conclusion is permissible, but it would appear that even where the globulin rise in this infection does occur, the percentage increase is not nearly so marked as that noted in some of the loop animals with complications or in those with abscess formation and tissue destruction.

TABLE X.

Serum Proteins in Dogs with Eck Fistula.

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein. constituents.	Protein quotient.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
48	Nov. 16	5.9	4.0	1.9	69	31	1.4	2.2	Sept. 13. Operation. Normal condition.
49	Dec. 28	5.7	4.2	1.5	74	26	1.6	2.8	Sept. 14. Operation. In excellent condition until Nov. 16. Moderate distemper and loss in weight. Poor appetite and gradual downward course. Definite intoxication on Dec. 28. Convulsions followed by death on Dec. 29. *Autopsy.*—Eck fistula perfect.
50	Nov. 22	7.3	4.2	3.1	58	42	1.5	1.3	Oct. 12. Operation. In excellent condition until Oct. 21. Development of distemper. Nasal discharge and cough. Condition on Nov. 22 not so good. Nose ulcerated.
51	Apr. 5	5.5	3.9	1.6	71	29	1.7	2.4	Feb. 22. Operation. In excellent condition on Apr. 5.
52	" 18	4.6	2.2	2.4	47	53	2.3	0.9	Mar. 10. Operation. Splenectomy on Mar. 23. Moderate loss in weight. Some vomiting and poor appetite for 10 days before date of blood examination.

Similarly, the blood of a number of Eck fistula dogs (Table X) was studied. Only one of the animals observed presented definite symptoms of intoxication at the time the protein partition was determined, and in this instance it was found normal. Another animal (Dog 52), on the other hand, showed a definite rise in globulins, but this animal had been in poor condition for 10 days prior to the blood examination. The low protein content obtained in this animal on this date is in keeping with the loss of weight and the impaired nutrition. Dog 50 also showed an increased globulin content, but this animal had developed distemper a month previously, and still had some signs of infection when the blood analysis was made.

DISCUSSION.

A satisfactory explanation of our experimental observations would require a knowledge concerning the origin and chemical relation of the various protein fractions, which we do not possess at present. It is permissible, however, to discuss several points on the basis of the data available. According to the work of some observers, the increase in globulins might be regarded as one of the phenomena which attend the development of the immunity or tolerance gradually acquired by an animal with long-standing obstruction or that following the repeated injections of proteose. Moll (7) has given some experimental evidence in support of such a view by showing that the formation of precipitins in the sera of animals injected with foreign protein parallels in a measure the rise in the globulins which occurs in them. Our experiments, however, do not give support to this interpretation. As already pointed out, we were unable to demonstrate that the development of resistance by an animal is accompanied by a heaping up of globulins in the blood. It will be recalled that following the repeated proteose injections in immunizing doses no increase in the serum globulins took place; whereas, in the loop animals in which this was a striking feature we were unable to show that the globulin increase was in any way related to periods of clinical improvement, or that a fall was associated with the development of a more severe intoxication.

It would seem more probable that the alteration in the normal

partition of albumins and globulins, and the complete inversion of the ratio following the rapid absorption of a toxic exudate is due to some fundamental change in metabolism. That such a metabolic upset occurs in the intoxications studied has been demonstrated by one of us (2). It has been shown that the exciting cause in some of these intoxications is the absorption of a toxic substance resulting from local tissue disintegration, or an upset in the delicate protein equilibrium. These poisons so formed are widely distributed in the body, and cause much protein injury. One result of this extensive destruction of body protein is the accumulation of non-coagulable nitrogenous substances in the blood, and an increased elimination of nitrogen immediately following the injury. It is, therefore, not unreasonable to assume that such a marked metabolic disturbance may also register a change in the coagulable proteins of the blood.

Still more difficult to explain, however, is the rapidity with which the inversion of the ratio is produced, and the increase of the globulins at the expense of the albumin fraction. From a theoretical viewpoint, a heaping up of blood globulins might be the result of a more rapid formation or a less rapid destruction of this substance or, lastly, of its diminished utilization. From what has already been said, it would seem that catabolism is especially active in these intoxications, and that anabolism is more or less at a low ebb. On the basis of the experiments now available, it is not possible to state with certainty which of the metabolic phases is more responsible for the changes observed. According to the work of Moll (8), the rise in the globulin content may be explained as the result of a more rapid conversion of albumin into globulin due to the accelerated metabolism; whereas the observations of Cervello (9) suggest the explanation that a heaping up of globulins may result from the diminished utilization of them by the body tissues.

It is worth while keeping two facts in mind. With an acute and profound intoxication of almost any type, we usually observe more or less increase of the globulin fraction at the expense of the albumin portion. With a slowly induced tolerance or immunity to a proteose or similar poison following repeated small doses of the poison, we usually observe little change, if any, in the globulin-albumin ratio. There is no relation between the tolerance or immunity developed

and the upset in the globulin-albumin fractions. There does seem to be a relation between the shock and intoxication caused by the poison and the globulin increase: in general, the more profound and acute the intoxication, the more does the globulin increase at the expense of the albumin, even to an inversion of the normal ratio.

These severe intoxications show a remarkable increase in the elimination of urinary nitrogen above the normal base-line level (Whipple, Cooke). There is strong evidence for profound tissue injury with an upset in the delicate protein balance of the body cells. May we assume that the change in the blood proteins is primary and dependent upon the direct action of the poison, or may we assume that the blood protein changes, like the blood non-protein changes, are dependent upon a primary cell protein reaction? More experimental work is required to answer these and many other questions concerning the blood proteins about which our knowledge is incomplete.

CONCLUSIONS.

The intoxication which develops as the result of a simple obstruction or a closed intestinal loop is accompanied by definite changes in the coagulable proteins of the blood serum. These changes consist essentially in an alteration in the normal albumin-globulin ratio; the globulin fraction is greatly increased and at times the normal relation of the two fractions may show a complete inversion.

The increase in the globulin content of the blood serum is most marked in the animals which show some of the complications met with in loop animals,—rupture of the loop and peritonitis. In the latter conditions especially, the globulin increase is rapid and large. We believe this reaction to be of diagnostic value in acute infections attended by the sudden liberation and absorption of a toxic exudate.

Infections and intoxications produced by inflammatory irritants are also accompanied by a rise in the blood globulins. This observation suggests that tissue disintegration with absorption of toxic products is responsible for the changes noted, and that bacterial invasion is important only in as far as it gives rise to toxic substances.

Animals which have developed a tolerance to proteose intoxication following the periodic injection of small doses of proteose do not

show a globulin increase. These experiments do not support the view that the rise in globulins observed in these experimental conditions is an expression of a resistance or tolerance developed by the animal.

From the experimental evidence it seems more probable that the alteration in the partition of the blood protein fractions is one of the results of the metabolic disturbance which has been shown to occur in these conditions.

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ON THE NYMPH AND PROSOPON OF THE TSUTSUGAMUSHI, LEPTOTROMBIDIUM AKAMUSHI, N. SP. (TROMBIDIUM AKAMUSHI BRUMPT), CARRIER OF THE TSUTSUGAMUSHI DISEASE.

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Plates 24 to 27.

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INTRODUCTION.

Tsutsugamushi or kedani disease is an acute exanthematous infectious disease, which at present occurs only in the northern coast districts of Japan; *i.e.*, in Niigata ken, Akita ken, and Yamagata ken. Recently it has also been found to exist in Formosa (1). The mortality of the disease varies, according to the district and year, between 20 and 50 per cent. It closely resembles Rocky Mountain spotted fever, but, as Ashburn and Craig (2) have pointed out, it possesses some distinct features. Among previous investigators of the disease may be mentioned Baelz, Kawakami, Tanaka, Kitasato, Ogata, Asakawa, Kitajima, Miyajima, Hayashi, Kawamura, Arima, Sakai, and Hattori, who have studied the etiological, pathological, and clinical features. The specific cause of the disease has not yet been established with certainty. Last year we undertook to investigate the disease, and especially the question as to its cause and means of transmission. As regards the specific germ we expressed the opinion that certain piroplasma-like forms discovered by us, which occur in the spleen, lymphatic glands, and in the blood, but not in the red corpuscles, and which may belong to the Sporozoa, may be the cause (3). We shall not describe them here in detail, but shall confine our report to the carrier of the disease, a species of Trombidium.

That tsutsugamushi disease is transmitted to man by a diminutive mite, is now established. Baelz (4), to whom we owe the first scientific description of the disease, was at first not inclined to admit this fact, but it was undeniably proven by later investigators through clinical observation and experiments on animals. This mite is called in Japan tsutsugamushi (disease mite), akamushi (red mite), kedani (hairy mite), or shimamushi (island mite), and is similar to the European harvest-bug, *Leptus autumnalis* (Baelz). Tanaka (5) was the first to describe it in detail. The specific determination was first undertaken in 1910 by Brumpt (6), who considered it to be a larva of a new trombidium, which he called *Trombidium akamushi*, apparently, however, without finding its nymph and prosopon. Recently Kawamura and Komagata (7) have published a detailed description of the external and internal structures of this mite. The studies mentioned above refer only to the larva of a hitherto unknown trombidium, *Trombidium akamushi* Brumpt, which attacks warm-blooded animals as well as man. The nymph and the prosopon of the tsutsugamushi, however, do not bite warm-blooded animals and have not yet been detected with certainty. Several authors claim to have discovered or bred the nymph or the prosopon, but their reports are not convincing. Some trombidium larvæ are well known in Europe and America under the names *Leptus autumnalis*, *Leptus irritans*, *Leptus americanus*, harvest-bug, redbug, chigoe, etc. They also bite human beings and are more or less closely related to the tsutsugamushi; the other larvæ, however, never cause so serious a disease as the latter. That the investigation of this mite has so far been neglected is chiefly due to the danger of the bite which causes the disease, and to which the investigators are exposed while working in the infected region.

Since beginning the present investigation, from November, 1915, until August, 1916, we have frequently been in the infected districts from 2 days to 1 week every month. In summer, when there is danger of being bitten by the mite, we dressed in such a way as not to expose any part of the body except the eyes; and we rubbed the body with some mite-killing agent, such as phenol, sulphur ointment, or insect powder. At other seasons, when there is usually no danger of being bitten, we could work without special precautions. Of the endemic regions in Japan we selected as a place for research the environs of Yachi on the banks of the Mogami river in Yamagata ken, which for the last 3 years has been known as an endemic district. We visited the district near Yuzawa in Akita ken once only. In the suburbs of Tokyo, where the tsutsugamushi is not found, and consequently the disease does not occur, we have on several occasions collected various trombidia. After investigations extending

over 1 year, we are now in a position to report on the nymph and the prosopon of the tsutsugamushi. We were often misled by the circumstance that in the endemic district various kinds of trombidia are found, which in contrast to the prosopon of the tsutsugamushi are more striking in size and color, and therefore more easily found than the prosopon of the tsutsugamushi. At first we mistook a particular species of trombidium for the parent of the tsutsugamushi, as other authors have also done both before and after us. Subsequently we found that there occur a number of species of trombidium in the endemic region and we have been able to isolate with certainty at least five species, which we have called provisionally Trombidia A, B, C, D, and E (8).

Trombidia A, B, C, and D lay their eggs in culture soil, and we were able to raise larvæ from the eggs. With Trombidium B we succeeded in raising nymphs from the larvæ. We also found the nymphs of Trombidia A, B, and C in nature. We shall not go into detail about the various species of trombidium, but merely point out that Trombidium B may be regarded as a variation of *Trombidium fuliginosum* Herm, and that Trombidium D cannot be distinguished, at least morphologically, from the European *Trombidium holosericeum* Linné. Trombidia A, C, and E are perhaps new species.

The larva of Trombidium B somewhat resembles the tsutsugamushi, and at first we believed them to be identical. In raising the eggs of Trombidium D we watched their development closely, because we thought that we might be able to raise tsutsugamushi from them, as *Trombidium holosericeum* has generally been considered to be the parent of *Leptus autumnalis* (Mégnin (9)), and the latter is so closely related to the tsutsugamushi that it may only be a variation of it, and as *Trombidium holosericeum* corresponds to Trombidium D. But since the larvæ obtained differed in essentials from tsutsugamushi and from *Leptus autumnalis*, we were forced to the conclusion, that *Trombidium holosericeum* Linné, is not the parent of *Leptus autumnalis*, as some authors have observed.[1] We believe that we have found the real parent of the tsutsugamushi, and we wish to report

[1] See Nagayo, M., Miyagawa, Y., Mitamura, T., and Imamura, A., Is *Trombidium holosericeum* the Parent of *Leptus autumnalis*, *J. Exp. Med.*, 1917, xxv, 273.

here on the nymph and the prosopon of the tsutsugamushi, which have hitherto remained unknown.

The Nymph of the Tsutsugamushi.

On April 30 and May 2, and 3, 1916, we received from Okiage and Nakagawara numbers of tsutsugamushi-carrying field mice. The lobes of their ears, which were thickly covered with the mites, were cut off and put into a glass dish. The mites then gradually loosened their hold and left the host, the fully fed ones sooner than the others. The mites were put in glass bottles, test-tubes, and glass dishes, which had been provided with moistened soil from the endemic districts, thoroughly sterilized by heat. On June 8, about 40 days later, three nymphs were found in one bottle, and later more (twenty-one in all) in the other vessels. Hayashi (10) and Kitajima and Miyajima (11) claim to have bred nymphs artificially before, but they do not describe the nymphs adequately, so that we cannot compare ours with theirs. We experienced no great difficulty in the culture of the nymphs. It is necessary to protect the soil from drying and rotting during the period of the experiment. The nymphs show great motility and like to hide in the ground. The second cultivation experiment, which we undertook with the larvæ taken from field mice caught on July 19, was likewise successful, and took a much shorter time than the first. After 10 days, on July 29, we found small nymphs crawling about, upon and in the soil of the vessels. The total number of nymphs produced amounted this time to over 80. They were a little smaller than those of the former breed, so it may be presumed that in the first experiment the nymphs were already some days old when they were discovered.

On July 30 we received two field mice from Nakagawara and for the third time we used the numerous attached tsutsugamushi for breeding the nymphs. For the purpose of studying the evolution of the pupa we distributed the well fed larvæ in eight breeding vessels, each of which contained 20 to 30 larvæ. After 3 full days, *i.e.*, on August 2, the first vessel was examined, and the others one by one on each succeeding day. This time the nymphs came out after 8 days, on August 7. The metamorphosis of the larva into the nymph

proceeds, though subject to slight variation, in general in the same manner as that described by Henking (12) for *Trombidium fuliginosum*. On the 4th day we already found the nymphophane. The oval immovable pupa is in every case distinguished by the formation of the so called *Apoderma* of Henking (the *Zwischenhaut* of Claparède (13)), lying under the larval cuticle. This is nearly transparent and supplied with single granule-holding round cells, which may, however, occasionally form groups (*Haemamoeben* of Claparède). Below this skin is the thin nymphal cuticle. The new extremities are already budding out and are likewise enveloped by the apoderm. There are now four pairs, the fourth pair being a new formation, as Henking has already described in *Trombidium fuliginosum*. The original three pairs of legs and the mouth apparatus have already undergone complete histolysis, so that nothing is visible under the original larval skin. The legs break off easily and are always outstretched, they never take the bent position, which is often found in dead larvæ. The original mouth apparatus is pushed somewhat forward and is easily detached from the body of the nymphophane. In the course of the following days the newly formed legs, of which the first pair is the best developed, grow longer and show a distinct arrangement of the parts and claws. They are directed obliquely backwards and towards the middle, so that the distal ends of each pair touch in the median line.

We have less to say of the development of the mouth apparatus. On the 4th day a small hemispherical protuberance is seen under the original mouth part of the larva, completely separated from the latter by the apoderm. Within this prominence one sees the anlage of the mouth parts; on the following days one can gradually distinguish the palpi, directed ventrally and backwards, and the mandibles between them. On the dorsal side of the head a peculiar spur-like structure is seen, with its point directed anteriorly. It projects from the chitinous skin of the nymph and reaches through the apoderm almost to the original larval skin. Henking does not mention this structure in *Trombidium fuliginosum;* it is probably an organ of the nymph to enable it to come out of the larval cuticle. In an 8 day old nymphophane we had excellent proof of this; we saw how the chitinous cuticle of the larva was pierced and torn by

this spur. The apoderm meanwhile increases in thickness and becomes provided with numerous acuminate excrescences. Later it grows thinner again, often becomes folded, and shows irregularly stratified lamellar structures. Through this apoderm the underlying newly formed hairs of the nymph are seen. The round cells, fairly numerous at one time, gradually disappear again. Shortly before the nymph emerges, the apoderm undergoes granular degeneration. Thus the evolution of the nymph is completed within a relatively short time, so that on the 9th day the majority of the new nymphs are found in or upon the soil of the receptacle.

Morphology of the Nymph.

Size and Form.—The nymph is a small, rather long, oval mite with eight legs, and shows between the cephalothoracic region and the abdomen a distinct constriction, especially conspicuous during life. The thorax and abdomen are correspondingly rounded at the sides, and can easily be distinguished. The length of the body from the points of the mandibles is 0.342 to 0.644 mm.; the maximum width of the cephalothoracic region is 0.168 to 0.280 mm.; and that of the abdomen 0.198 to 0.324 mm.

TABLE I.

Measurements of the Legs of a Nymph 0.612 Mm. in Length.

	Total length.	Joint.					
		1	2	3	4	5	6
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1st pair.							
Length	0.432	0.028	0.060	0.060	0.072	0.088	0.124
Breadth		0.036	0.036	0.046	0.052	0.060	0.060
2nd pair.							
Length	0.236	0.023	0.028	0.028	0.040	0.040	0.068
Breadth		0.036	0.040	0.036	0.034	0.028	0.024
3rd pair.							
Length	0.208	0.024	0.024	0.024	0.028	0.044	0.064
Breadth		0.040	0.028	0.032	0.028	0.024	0.020
4th pair.							
Length	0.284	0.040	0.040	0.028	0.044	0.052	0.080
Breadth		0.036	0.032	0.032	0.028	0.028	0.024

Color.—The body is grayish white or pale yellow, while the legs are colorless. The contents of the stomach shining through the body give it the yellow color. The hairs and epidermis are colorless.

Legs.—There are four pairs of legs. The first pair is the longest, the fourth pair coming next, then the second and third (Table I).

The anterior pair is by far the longest; the segments, in contrast to the other legs, become longer and broader towards the distal end. Each tarsus is provided with two claws but has no clinging hairs. The claws of the anterior pair are much shorter (length 0.016 mm.) and less curved than those of the other legs (0.022 mm.).

Epimeres.—The epimeres of the first and second pairs of legs of the same side lie close together, as do those of the third and fourth pairs, though nowhere grown together, so that they form on each side an anterior and a posterior group. The surface of the epimeres is finely punctated. The first pair of epimeres extends distally for about one-third of their length over the edge of the body.

Measurements of the Epimeres.

	Leg.			
	1	2	3	4
	mm.	*mm.*	*mm.*	*mm.*
Length	0.080	0.060	0.068	0.076
Width	0.044	0.048	0.052	0.048

	mm.
Distance between the anterior and posterior groups	0.048
Transverse distance between the pairs of the anterior groups	0.020
" " " " " " " posterior "	0.060

Tracheal and Stigmatal Plate.—We have not yet found with certainty the tracheal openings which are distinctly visible in the larvæ on the first pair of the epimeres. At least the scaly chitinous structure surrounding the tracheal openings, Henking's *Stigmenschutzapparate*, which is distinguished in the nymphs and prosopa of other trombidia, is not distinctly developed in ours.

Genital Opening and Anus.—The genital opening is oval and is situated in the median line of the ventral side of the abdomen, behind the fourth epimeres. The anus opens further back.

	Genital opening.	Anus.
	mm.	*mm.*
Length (sagittal diameter)	0.072	0.056
Width (cross diameter)	0.048	0.040

The genital opening is surrounded on either side by a raised portion, provided with two flat oval plates, sucking discs.

Mouth Apparatus.—The palpus consists of four joints besides the basal part. The second is the largest in size. The terminal joint is provided with a claw (length 0.024 mm.) and a longer appendage, the thumb (length 0.036 mm.). This appendage is not club-shaped, as is usual with trombidia, but tapers towards the end and is covered with numerous pinnate hairs. If we regard this appendage as an independent joint of the palpus, as is done by Banks (14), Fiebiger, and others, we may count five joints in the latter.

Size of the Palpi.

	Joint.			
	1	2	3	4
	mm.	*mm.*	*mm.*	*mm.*
Length	0.016	0.048	0.036	0.034
Width (greatest)	0.020	0.044	0.032	0.020

Mandibles.—The mandible, which averages 0.112 mm. in length has at the front a sharp, dorsally curved hook (length 0.052 mm., width 0.020 mm.), with fine teeth on the concave edge. Both mandibles are imbedded in a boat-shaped area, which gradually tapers towards the front and is pilose. The mandibles are covered at the posterior part by a corrugated epithelium, an elongation of the dorsum of the body, the epistome. A little in front of this epistome there is one hair, feathered and pointed, 0.0334 mm. long, in the median line between both mandibles.

Scutum.—The scutum consists of two symmetrical circular parts (the distance between them 0.020 mm.), each bearing a long hair (length 0.100 mm.), and a middle part connecting the two, which extends behind into a short process and forward into a hairless chitinous crista reaching to the end of the epistome.

Eyes.—The eyes are not easily visible. They are sessile and situated close to the pseudostigma of the scutum. The eye contains

fine reddish granules, but is on the whole rudimentary, compared with that of the larva, and is not provided with clearly developed lenses.

Hairs.—The paired tactile hairs of the scutum have already been mentioned. The entire body of the nymph is thickly covered with hairs. The most striking are those on the back of the abdominal segment, which are longer than the others (0.056 to 0.080 mm.), and have a rather thick shaft and club-shaped end. The surface of the shaft is covered with innumerable small pinnate hairs. The clubbed shape of the end varies in different individuals, and probably depends on the nourishment and state of development of the nymph. The length of the hair, too, shows individual variations. These hairs seem to be peculiar to the nymph of the tsutsugamushi, and have so far been found besides only in *Trombidium holosericeum* among our trombidium species. They differ from the latter in that they are colorless, while the club-shaped hairs of *Trombidium holosericeum* are red. They arise from distinctly developed hair follicles. In other parts of the body the hairs are generally shorter (0.028 to 0.050 mm.) On the anterior part of the cephalothoracic region, especially before the scutum, there is little hair. The hairs of the legs and mouth apparatus are not clubbed but simply pinnate and pointed, and on the whole similar to the pinnate hairs of *Trombidium fuliginosum* and our Trombidia B and C. The hairs are especially numerous on the first pair of legs and on the appendage of the palpus.

The Prosopon of the Tsutsugamushi.

Encouraged by the result of breeding the nymphs, we tried to find them in the endemic district, and succeeded after great difficulty. On July 13 we found 3 specimens in Horiguchi, 8 and 7 respectively on July 17 and 18 in Okiage, 7 on July 19 in Nakagawara, 1 on July 31 in Horiguchi, 15 on August 1 in Nakagawara, 1 on August 9, and 23 on August 14, making a total of 65 specimens. It requires great patience to search in the oppressive heat of summer, with the entire body carefully covered against the larval bite, for this tiny pale colored insect which can be distinguished from a grain of sand only by its movement. We often came home without results. One

day, when five persons took part in the hunt, only one specimen was found. The tsutsugamushi are usually found in the ground, and when brought to light by the turning of the soil, they try to hide again in the earth. Their color is sometimes grayish white without any yellowish red shade, but usually pale yellow or orange. In the latter case the hairs remain colorless. The body of the animals is always considerably larger than that of artificially bred nymphs as is shown in Table II.

TABLE II.

No.	Length.	Greatest width.		Color.
		Thorax.	Abdomen.	
	mm.	*mm.*	*mm.*	
1	1.166	0.564	0.720	Pale yellow.
2	0.738	0.415	0.476	Gray.
3	0.918	0.424	0.600	"
4	0.922	—	—	Orange-yellow.
5	About 1.000	—	—	Pale yellow.
6	" 1.000	—	—	Orange-yellow.
7	0.886	0.416	—	Gray.
8	1.152	0.612	—	"
9	0.810	0.450	0.486	Pale yellow.
10	1.080	0.540	0.700	" orange.
11	1.250	0.630	0.900	Orange.
12	1.260	0.620	0.810	"
13	0.950	0.584	0.680	Grayish white.
14	1.116	0.504	0.648	Pale orange.
15	0.990	0.396	0.414	Stained.
16	1.440	0.720	0.900	Orange.
17	1.260	0.594	0.756	"
18	1.170	0.504	0.648	"
19	1.440	0.840	1.080	Pale orange.
20	1.260	0.680	0.936	Orange.

When we first discovered these mites we were of the opinion that we had before us the free living nymphs of the tsutsugamushi, for we found with the exception of the difference in size and a deeper coloring in many specimens no essential difference in outward appearance from the artificially reared ones. But when we made a minute comparison, we found a difference in the number of sucking discs

of the genital opening. While the nymphs, as already described, always have two pairs, we counted in this case three pairs of discs. Banks (14) has already pointed out that in certain mites, especially in Hydrachnidæ which resemble Trombidia, the shape and number of the sucking discs are important characteristics in distinguishing the species. We found in Trombidia A and B always three pairs, in the nymph as well as in the prosopon, while Trombidium C has only two in the nymph and three in the mature animal.

It therefore seems that the animals caught by us were not nymphs but mature animals. We have hesitated to pronounce them as such until we succeeded in finding eggs in their body. In animals caught on July 31 and August 1 we found eggs in various stages, which could be distinctly seen by pressing out the contents of the stomach under a cover-glass. The mature eggs are round, and have a relatively thin shell and an almost colorless yolk; their diameter is about 0.162 mm. Besides these a few immature, mostly smaller eggs can be seen through the skin. But we did not find any prosopa rich in eggs. It is probable that they deposit their eggs only in small quantities; at any rate, it seems that they never deposit a single heap formed of hundreds of eggs, as is the case with other trombidia.

Measurements of the various parts of a prosopon 1.166 mm. long are given in Table III.

TABLE III.

	mm.
Width of thorax	0.504
" " abdomen	0.720
" " constricted part	0.468
Distance of the third pair of coxæ (epimeres)	0.120
Length of mandibles	0.180
Width " "	0.068
Length " mandible hook	0.068
Total length of palpi with basal joint	0.344

TABLE III—*Continued.*

Palpi.

		mm.
Basal joint.	Length	0.120
	Width	0.042
Joint 1.	Length	0.024
	Width	0.040
" 2.	Length	0.084
	Width	0.044
" 3.	Length	0.056
	Width	0.040
" 4.	Length	0.060
	Width	0.024
Claw		0.020

Leg.

		1	2	3	4
		mm.	*mm.*	*mm.*	*mm.*
Coxa.	Length	0.120	0.080	0.112	0.112
	Width	0.056	0.048	0.090	0.090
Joint 1.	Length	0.064	0.048	0.044	0.052
	Width	0.048	0.044	0.040	0.044
" 2.	Length	0.056	0.048	0.048	0.060
	Width	0.048	0.040	0.038	0.042
" 3.	Length	0.088	0.048	0.044	0.060
	Width	0.060	0.040	0.038	0.044
" 4.	Length	0.108	0.060	0.056	0.068
	Width	0.060	0.044	0.038	0.040
" 5.	Length	0.112	0.064	0.068	0.088
	Width	0.064	0.044	0.038	0.040
" 6.	Length	0.200	0.108	0.104	0.132
	Width	0.072	0.072	0.034	0.036
Claw		0.028	0.036	0.033	0.032
Total length of legs without epimeres		0.628	0.378	0.364	0.460

		mm.
Width of dorsal shield		0.072
Greatest sagittal diameter of dorsal shield		0.038
Length of tactile hair		0.120
Genital parts.	Length	0.113
	Width	0.080
Genital opening.	Length	0.060
	Width	0.036
Sucking discs.	Length	0.028
	Width	0.036
Anus.	Length	0.048
	Width	0.056
Distance between anus and genital opening		0.080
Length of dorsal hairs		0.056–0.068
" " hairs of legs		0.032–0.042

Morphologically the prosopa of the tsutsugamushi, differ greatly from other trombidia. Their chief characteristics are their small size, pale color of body, indistinct eyes and stigma. They are also characterized by the distinct constriction between the cephalothoracic region and the abdomen and the peculiarity of their hairs.

Sections have so far been made of six nymphs and twelve mature animals. Except for the genital gland both forms show in their inner morphology practically no differences. They have, compared with other species, a comparatively soft chitinous coat, so that for making serial sections a short immersion in alcohol or ether is sufficient. Both forms have a large brain and a lobed stomach. The intestine, esophagus, fat bodies, etc., are like those of the other trombidia. The large paired salivary glands possess several lobes and show three different kinds of cells according to their protoplasm or enclosed granule. One variety has fine granules which stain weakly with eosin, and the second coarse, distinctly eosinophilic granules, while the third consists of almost transparent cells filled with numerous vacuoles. Occasionally all three kinds of cells are found in the same lobe. At least two ducts are visible, which later unite. The Malpighian tubule consists of a pair of tubes. Both ends lie on the ventral side of the salivary gland and genital organ, the middle part being situated near the brain and the stomach.

The tracheal system is extraordinarily rudimentary, as are the eyes.

We were unable to find any tracheal capillaries, while in Trombidia A, B, C, and D numerous tracheas are easily seen in almost all parts of the body.

Regarding the genital gland, we found in the artificially bred nymphs only cell heaps directly behind the brain, between the genitalia and the stomach, which may be regarded as the undifferentiated germ cells. The animals caught in the open, on the contrary, show a well developed gland; *i.e.*, testicles or ovaries. In the female there is an oviduct on either side and a uterus provided with a muscular wall. The ovary contains ova in various stages of development. In three cases we found eggs already enclosed in the shell.

According to the external and internal morphological structure of the prosopa there is no possible doubt that we have before us the mature form of the tsutsugamushi.

We should mention that our nymph of the tsutsugamushi is closely related to that of *Leptus autumnalis*, artificially bred by Brandis (15), while the prosopon of the tsutsugamushi is different from *Trombidium holosericeum;* the latter, however, has, since Mégnin, often been considered the parent of *Leptus autumnalis*.[1]

Cultivation of the Larva of the Tsutsugamushi.

After we had discovered the prosopon of the tsutsugamushi in the open field (16), we tried to breed larvæ (tsutsugamushi) from the mature animal. To this end we distributed many prosopa into a number of small glass vessels and examined each vessel every 2nd day. On August 18 we found for the first time three small, yellowish red insects crawling about on the side of one of the vessels, which after being fixed in Berlese's fluid were found to be tsutsugamushi larvæ. The next day four larvæ were discovered in another vessel. No eggs were found in the soil of the two vessels, one of which on previous examination had been found free of trombidium eggs, while the other had been sterilized by heat. In the investigation of Trombidia A, B, C, and D we had found heaps consisting of hundreds of eggs. In one of the vessels we found the dead bodies of two prosopa. The hatched larvæ are undoubtedly the true tsutsugamushi; *i.e.*, they show no essential difference from tsutsugamushi larvæ taken from field mice and man; but they are distinctly smaller than those that have fed on the lymph of warm-blooded animals; they appear almost round in contrast to the more oval shape of the latter. Moreover, larvæ which have not yet sucked on mammals, have a broader mouth part and a more deeply colored body, which is orange-red in appearance. Otherwise the palpi, legs, epimeres, hairs, stigmata, eyes, and the appearance of the body are alike in both. Though we have not yet found eggs in the soil of the receptacle, it seems certain that the hatched larvæ are the offspring of the prosopa, because the soil had been thoroughly sterilized by heat before the latter were put in. There are two possibilities as to the way in which the larvæ develop; either the prosopa deposit so few eggs that they easily escape the attention of the observer, or no eggs are deposited, the larvæ leaving the body of the parent already formed

(viviparous). At any rate, by the artificial breeding of the larvæ the parent of the tsutsugamushi has been definitely established, and the tsutsugamushi is disinct in this as it is in the nymph and prosopon stages.

SUMMARY.

Nomenclature.

As mentioned above, the prosopon and the nymph of the tsutsugamushi have many characteristics which distinguish it from the other trombidia. These are the pale color and small size of the body, delicacy of the skin, the conspicuous constriction between the cephalothoracic region and the abdomen, rudimentary eyes and tracheal openings, absence of tracheal capillaries, etc. The fact that the thumb or the appendage of the palpus does not start from the side of the fourth joint of the latter but from its end, is another characteristic which does not occur in other trombidia. From the biological point of view two facts should be mentioned; *i.e.*, the adult female does not deposit eggs in heaps, and in contrast to those of other species, the larvæ hatch out all through the year and feed on mammals. From these characteristics running through all the stages of development, we believe that we are justified in claiming a new genus for the tsutsugamushi. We therefore propose to change the scientific name given to this mite by Brumpt in 1910, *Trombidium akamushi*, and to introduce instead, according to the suggestion of Drs. Goto and Watase, the name *Leptotrombidium akamushi*.

Determination of Leptotrombidium akamushi, N. Sp.

Prosopon and Nymph.

1. Pale color.
2. Delicacy of the skin.
3. Distinct constriction between the cephalothoracic region and the abdomen.
4. Rudimentary, sessile eyes.
5. Indistinctness of tracheal openings.
6. Absence of tracheal capillaries.
7. Crista abruptly widened at the end.
8. One palpal claw.

Larva.

1. One scutum.
2. Round spiracles.
3. Legs, six joints, not counting the coxa.
4. Hairs on the coxa: one each on Joints 1, 2, and 3.
5. Hairs on the scutum, including two tactile hairs, seven; one is in the median line.

Specific Determination of Leptotrombidium akamushi, N. Sp. (Trombidium akamushi Brumpt, 1910).

Prosopon.

1. Small size.
2. Three pairs of sucking discs on the external genitalia.
3. Hairs of the body feathered, club-shaped at the end, and colorless.
4. The appendage of the palpus tapers distinctly towards the end.

Nymph.

1. The size of the body is smaller than that of the prosopon.
2. Two pairs of sucking discs on the external genitalia.

There are no other distinct differences in the external morphology between the prosopon and the nymph.

Larva.

1. The color of the body is orange-red, but becomes paler after sucking on mammals.
2. The hairs of the body average 110 to 120 in number.
3. One pair of double eyes.
4. The sucking tube is visible when feeding on mammals (Hayashi).
5. The salivary gland is relatively large (Kawamura and Komagata).
6. The number and arrangement of the hairs on the legs and mouth parts correspond to the description given by Hirst.
7. The larvæ are found all through the year.
8. The larva is the carrier of tsutsugamushi disease in man.

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16. Nagayo, Miyagawa, Mitamura, and Imamura, On the Nymph and the Adult of the Tsutsugamushi, *Iji Shimbun*, 1916, No. 956.

EXPLANATION OF PLATES.

PLATE 24.

FIG. 1. Tsutsugamushi larva artificially bred. × 143.

FIG. 2. Tsutsugamushi larva from man, fully fed. × 135.

FIG. 3. Tsutsugamushi nymphophane on the 2nd day of breeding. × 79.

FIG. 4. Tsutsugamushi nymphophane on the 8th day of breeding. × 79.

FIG. 5. Tsutsugamushi nymph artificially bred from larva, seen from the ventral side. × 79.

PLATE 25.

FIG. 6. Tsutsugamushi nymphophane on the 7th day of breeding. The original larval skin is pierced by a spur-like structure of the nymphophane (left side upwards). × 220.

FIG. 7. Tsutsugamushi nymph artificially bred from larva. × 200.

PLATE 26.

FIG. 8. Tsutsugamushi prosopon (captured specimen). × 81.

FIG. 9. Tsutsugamushi. Sagittal section of a male prosopon. × 97.

PLATE 27.

FIG. 10. Tsutsugamushi. Sagittal section of a female prosopon. × 100.

FIG. 11. Field work in Okiage, one of the endemic districts in Yamagata ken. July 18, 1916.

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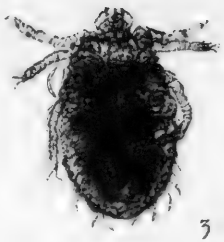

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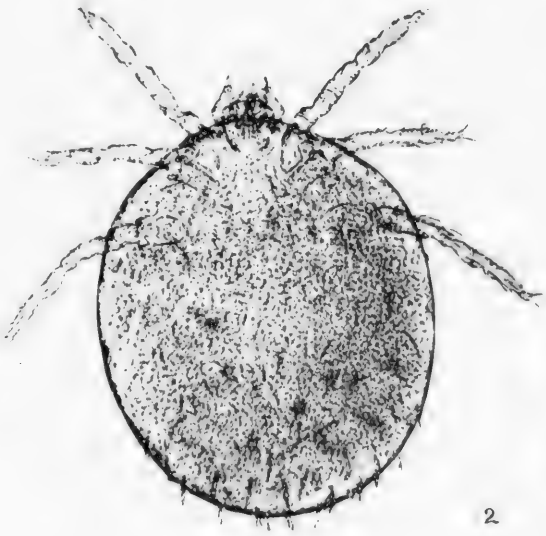

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(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)

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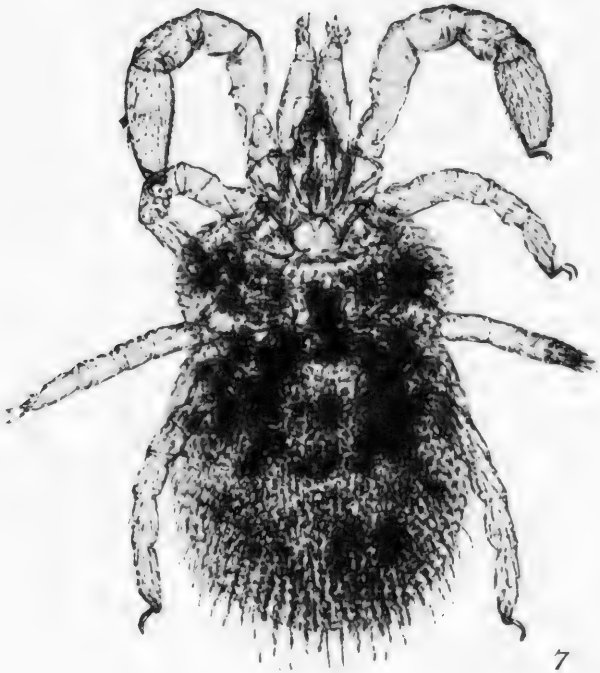

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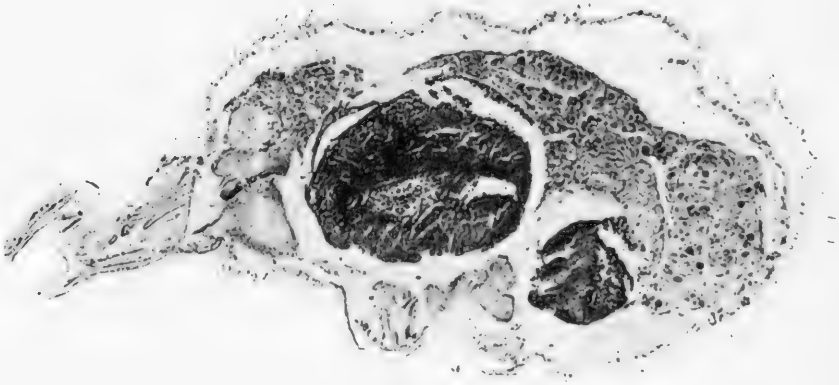

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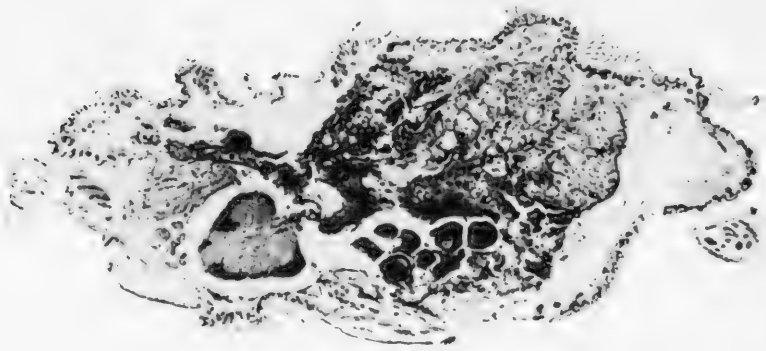
(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)



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(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)

IS TROMBIDIUM HOLOSERICEUM THE PARENT OF LEPTUS AUTUMNALIS?

By MATARO NAGAYO, M.D., YONEJI MIYAGAWA, M.D., TOKUSHIRO MITAMURA, M.D., and ARAO IMAMURA, M.D.

(*From the Imperial Institute for Infectious Diseases, Tokyo, Japan.*)

Plate 28.

(Received for publication, October 11, 1916.)

The question whether *Trombidium holosericeum* Linné, is the parent of *Leptus autumnalis* Latreille, (*Leptus autumnalis* Shaw) does not affect our work on tsutsugamushi directly. But indirectly, in the investigation of the life cycle of the tsutsugamushi, the carrier of tsutsugamushi disease, the question is of importance, because the tsutsugamushi, or akamushi, is almost identical with the European *Leptus autumnalis*. From our observations and from a study of the literature we cannot accept the view that *Trombidium holosericeum* is the parent of *Leptus autumnalis*.

Mégnin (1) first stated that *Trombidium holosericeum* was the parent of *Leptus autumnalis*, the latter having been studied by Latreille, de Geer, Gervais, and others before him. He observed that *Trombidium holosericeum* first appeared on the ground in April and was laden with numerous eggs from the end of May till June. He further succeeded in raising larvæ from these eggs, which according to him were *Leptus autumnalis* or the redbug. Mégnin does not state whether his morphological description of the larvæ is made from the larvæ artificially bred from *holosericeum* eggs. But it seems to us probable that Mégnin described the larva only from *Leptus autumnalis* which he had taken from warm-blooded animals, such as the rabbit, dog, etc., but not the bred *holosericeum* larvæ. In any case a close morphological comparison seems necessary for the identification of the larvæ, for it seems to us possible that Mégnin's artificially bred larva may have belonged to another species of trombidium.

When we first saw the larva of Trombidium B[1] bred from eggs we

[1] Nagayo, M., Miyagawa, Y., Mitamura, T., and Imamura, A., On the Nymph and Prosopon of the Tsutsugamushi, *Leptotrombidium akamushi, N. Sp.* (*Trombidium akamushi* Brumpt), Carrier of the Tsutsugamushi Disease, *J. Exp. Med.*, 1917, xxv, 255.

had no doubt that it was the tsutsugamushi, and that thus the parent of the latter was established. Later, however, we perceived inconspicuous differences, which nevertheless were essential differentiating characteristics between the two larvæ. The larva of Trombidium B is distinguished from the tsutsugamushi by the much smaller number of hairs, the localization and arrangement of the latter, the shape of the stigmata and of the mouth apparatus, as well as the length and the number of joints of the legs, etc. Mégnin does not give a detailed comparison but his statement that the *holosericeum* larva is identical with *Leptus autumnalis* has, nevertheless, been frequently cited. As far as we know, this statement has not been confirmed by reinvestigation; that is, by breeding *Leptus autumnalis* from the eggs of *Trombidium holosericeum*. Even Brandis (2), who found *Leptus autumnalis* in great numbers at Nietleben near Halle a. S. and for the first time was able to breed the nymph of the latter, does not mention the mature animal or eggs. Perhaps he did not succeed in finding *Trombidium holosericeum* or its eggs in the place where he had found many larvæ. His successful breeding of the nymph, which according to his sketches and description, closely resembles our tsutsugamushi nymph, is of great importance but this is not proof of Mégnin's statement. We have found since April, 1916, fifteen specimens of a trombidium species, which we have provisionally named Trombidium D, on the ground in various parts of Yamagata ken. Morphologically and biologically they are identical with *Trombidium holosericeum*. Their color is carmine red and they show the typical silky luster. They are 2.34 to 3.50 mm. long and 1.80 to 2.70 mm. broad. This trombidium is somewhat square, with a sagittal incision in the hind end of the body and with several grooves in the back. The hairs are long and pinnate, with clubbed ends; in the joints of the legs they are a little longer. The legs have two claws, without clinging hairs. The eye has a long peduncle. Since May these animals have laid several heaps of eggs in the vessels. The eggs measure from 0.15 to 0.18 mm., usually 0.176 mm. They are almost spherical, pale yellow, and shining, and gradually assume an orange-yellow color. Several larvæ appeared at the end of 3 weeks in the incubator at 22°C. These larvæ have a mouth apparatus somewhat resembling that of *Leptus autumnalis* and of tsutsu-

gamushi (Figs. 1 and 2), but there are also great differences, as the following table shows.

Larva of Trombidium D (*Trombidium holosericeum*).		Tsutsugamushi.	*Leptus autumnalis.*
Hairs of the body.	Not numerous (about 48); 2 pairs of long hairs at the hind end.	Numerous (about 110 to 120); all about equally long.	Numerous; all about equally long.
Dorsal shield.	Two.	One.	One.
Stigma.	Oval.	Round.	Round.

It is not necessary to go into more detail, as the points mentioned show that the larvæ of Trombidium D, *i.e.*, *Trombidium holosericeum*, are different from both *Leptus autumnalis* and tsutsugamushi. In Oudeman's classification, the larva of Trombidium D belongs to Allotrombidium, and the two other larval forms to Trombidium, according to the number of dorsal shields.

Mégnin further observed that *holosericeum* larvæ immediately after hatching parasitized on warm-blooded animals as well as on insects. He states: The larva, which is almost spherical when it emerges, and much less active than that of *Trombidium fuliginosum*, and with shorter claws, is the same as the common redbug, *Leptus autumnalis*, which as soon as it is hatched attaches itself to some animal or insect. If this is the case, Mégnin's *holosericeum* larva cannot be regarded as a genuine *Leptus autumnalis*, because Brandis (2), as well as others, has made different observations. Brandis says that if the mites are shaken by a human being or a warm-blooded animal they immediately attach themselves to it.

There is perhaps no mammal, which comes within their reach, unmolested by them; they have been found on hares, rabbits, various kinds of mice, badgers, hedgehogs, molebat, shrew, dogs, and cats. On birds, reptiles, insects, and spiders, I could, however, not effect any infestation, though on insects and spiders near relations of *Leptus autumnalis* parasitize.

Our tsutsugamushi attacks field mice, rabbits, guinea pigs, monkeys, and other mammals, but not insects. They are biologically and morphologically closely related to *Leptus autumnalis*, though recently some minor differences in the form of the dorsal shield, in

the hairs of the palpi, etc., have been emphasized by Hirst (3). The behavior of the two nymphs is also similar, as mentioned above. For these reasons we cannot accept the statement that *Trombidium holosericeum* is the parent of *Leptus autumnalis*. The true parent of *Leptus autumnalis* must be another species, which, as far as we can find in the literature, has not been described as yet. We believe that the prosopon of *Leptus autumnalis* must be similar to that of tsutsugamushi.

A detailed report on the nymph and the prosopon of the tsutsugamushi was given in the preceding paper,[1] to which we may refer the reader.

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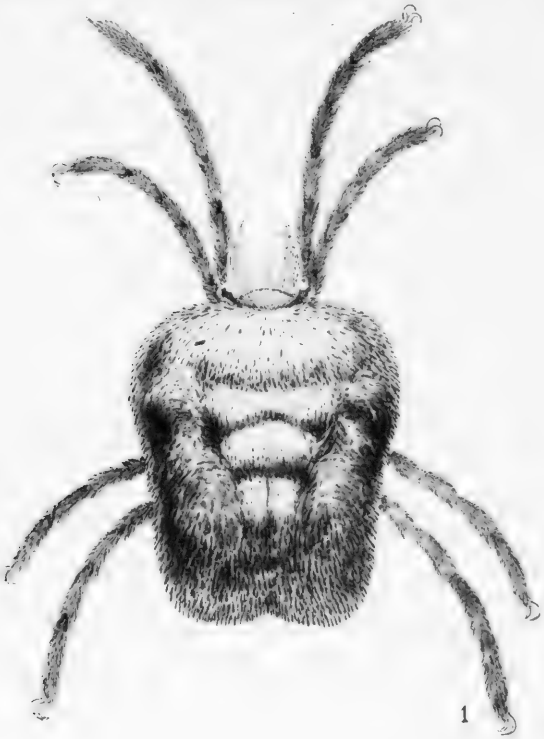
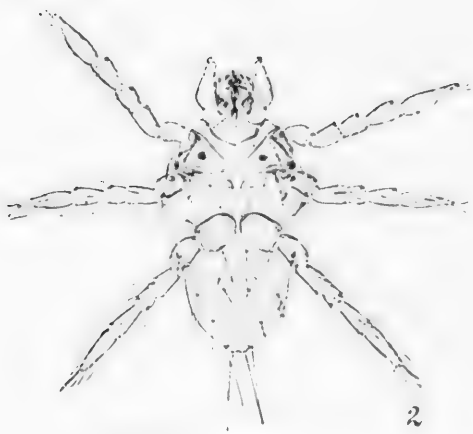
4. Hirst, On the Tsutsugamushi (*Microtrombidium akamushi* Brumpt), *J. Econ. Biol.*, 1915, x, 79.

EXPLANATION OF PLATE 28.

FIG. 1. Trombidium D (*Trombidium holosericeum*), adult. × 20.
FIG. 2. Trombidium D, larva hatched from egg. × 150.





(Nagayo, Miyagawa, Mitamura, and Imamura: *Trombidium holosericeum*.)

A CONTRIBUTION TO THE STUDY OF PANCREAS INTOXICATION.*

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(Received for publication, October 17, 1916.)

Two main views are held regarding the nature of the toxic agent of the autolyzing pancreas. One identifies it with active trypsin,[1] the other with split products of protein resulting from trypsin digestion.

Lattes[2] has recently shown that trypsinogen may be activated by some substance present in the dead pancreas cells, so that trypsin digestion may take place fairly rapidly in the autolyzing gland. This fact might of course be cited in favor of either of the two views, though Lattes himself is inclined to consider protein split products the active toxic agent.

Petersen, Jobling, and Eggstein[3] have shown that in acute intoxication from experimental hemorrhagic pancreatitis, the non-coagulable nitrogen of the blood is increased, indicating the absorption of material of a protein nature. Whipple[4] has isolated from the pancreas in which an acute experimental hemorrhagic necrosis had been produced, a toxic proteose which he considers an active agent in this disease.

The following study was undertaken[5] with the idea of purifying as far as possible the toxic part of the autolyzing gland. It was found that fresh pancreas extract in salt solution is toxic, and when injected intravenously produces symptoms of poisoning analogous

*Aided by a grant from The Rockefeller Institute for Medical Research.

[1] von Bergmann, G., and Guleke, N., Zur Theorie der Pankreasvergiftung, *Münch. med. Woch.*, 1910, lvii, 1673.

[2] Lattes, L., Über Pankreasvergiftung, *Arch. path. Anat. u. Physiol.*, 1913, ccii, 1.

[3] Petersen, W., Jobling, J. W., and Eggstein, A. A., *J. Exp. Med.*, 1916, xxiii, 491.

[4] Whipple, G. H., *J. Am. Med. Assn.*, 1916, lxvi, 2031.

[5] This investigation was begun with Dr. G. H. Whipple, at the Hunterian Laboratory of the Johns Hopkins Medical School.

to those resulting from duodenal loop fluid, and other toxic materials of a protein nature. The symptoms, however, develop relatively slowly, and reach their maximum about 2 or 3 hours after injection.

Method of Preparing Fresh Extract.

The pancreas is removed from a dog with aseptic precautions. It is cut finely and ground in a mortar with clean sand. 50 cc. of normal salt solution are added and mixed thoroughly with the macerated gland by grinding. The material is then filtered through gauze and cotton. The filtrate injected slowly intravenously into a dog causes a rapid fall in blood pressure with a more gradual but prompt return to normal or above. The clotting time of the blood is delayed and there is a profound fall in leukocytes. In from 1 to 3 hours after the injection, symptoms of extreme intoxication appear. These are persistent vomiting of bile-stained mucus; profuse diarrhea which becomes bloody; often bleeding from the gums; fall in blood pressure and in body temperature; slow, deep respiration; weak, rapid heart beat; coma and death if the dose is sufficient. If the dose is not lethal, the animal rapidly recovers, the temperature and leukocytes rise to above normal, vomiting and diarrhea subside, so that within 12 hours after the injection, the animal may appear normal, after a very severe intoxication.

Autopsy upon a fatally poisoned animal reveals extreme splanchnic congestion, especially apparent in the upper portion of the small intestine, the mucosa of which is deep purple in color from congestion and hemorrhage. Numerous focal necroses or hemorrhages are present in the liver. The gall bladder is edematous. The stomach and intestine are filled with bile-stained juices and mucus. By this method about one fatal dose may be obtained from a gland, for a dog of the same weight as the one from which the pancreas was obtained.

Treated rapidly in this manner, there can be little if any active trypsin present in the injected material. It seemed probable, therefore, that trypsin was not the active toxic agent, but some other substance present in the normal gland. Experiments with this idea in view revealed the fact that the β-nucleoprotein fraction of the gland is toxic and produces reactions identical with those following injection of the salt solution extract.

Preparation of β-Nucleoprotein from Pancreas.[6]

Fresh pancreas is chopped finely and ground in a mortar with sand, thoroughly stirred with twice its weight of distilled water, and heated to the boiling point for 10 minutes. The interstitial fluid is poured off and filtered, while hot, through ordinary filter paper, and the residue is again heated to boiling with one-half the original volume of water. This is filtered hot and the residue squeezed in gauze. The two filtrates are united. The fluid is opalescent. It is again filtered, and acidified until it contains 1 per cent acetic acid. A creamy white flocculent precipitate forms. It is cooled in running water and the precipitate completely thrown down by adding an equal volume of 95 per cent alcohol. It is placed in the ice box for 6 to 12 hours, and the supernatant fluid is then filtered or centrifuged off. The precipitate is redissolved in distilled water, made slightly alkaline with dilute sodium carbonate, and reprecipitated by rendering it slightly acid with dilute acetic acid. This process is repeated several times to get rid of soluble impurities. The precipitate is then washed with alcohol and ether, and dried in a desiccator. Of this material 0.1 gm. per kilo produces a profound intoxication and sometimes death in young animals. It is dissolved in 10 or 20 cc. of very dilute sodium carbonate and injected intravenously.

The fact that the β-nucleoprotein fraction of the dog's pancreas is toxic proves the presence of a poisonous material in the fresh gland which cannot be active trypsin since the substance is thermostable. The β-nucleoprotein, however, contains, besides its protein constituents, guanylic acid.

A pure preparation of guanylic acid[7] from yeast nucleic acid was found to be non-toxic for dogs in doses of 0.1 gm. per kilo when injected intravenously. Although the guanylic acid prepared by Bang[8] from beef pancreas was toxic for dogs, he was no doubt working with a relatively impure product.

[6] Steudel, H., and Brigl, P., Über die Guanylsäure aus der Pankreasdrüse, II, *Z. physiol. Chem.*, 1910, lxviii, 40.

[7] This material was obtained through the kindness of Dr. W. Jones.

[8] Bang, I., Chemische und physiologische Studien über die Guanylsäure, *Z. physiol. Chem.*, 1901, xxxii, 201.

It seemed from these experiments that the toxic material of the normal pancreas is the protein constituent of β-nucleoprotein, or is in some way associated with it.

When fresh dog pancreas was autolyzed for 12 to 24 hours with chloroform and toluene at 37°C., the autolysate was found to be toxic, though less so than the fresh gland, and after autolyzing for longer periods the toxicity disappeared. An attempt was then made to recover the toxic fraction from the autolyzed gland, and by this method a product has been obtained which is more toxic than the β-nucleoprotein.

Preparation of Toxic Material from the Autolyzed Pancreas.

Ground fresh pancreas is mixed with twice its weight of distilled water made slightly alkaline to litmus with sodium carbonate. About 1 cc. of chloroform is added and the mixture covered with toluene. It is placed in the thermostat and digested at 37°C. for 12 to 24 hours, being shaken at intervals. It is then made acid to litmus with dilute acetic acid, boiled for 10 minutes, and filtered hot through an ordinary filter paper. The clear yellow filtrate is made acid up to 2 per cent acetic, cooled in running water or in the ice box, and left to stand for several hours. A flocculent precipitate forms. This is centrifuged and the supernatant, slightly cloudy fluid is decanted. The precipitate is redissolved in dilute alkali and reprecipitated by acidifying with a few drops of 1 per cent acetic acid. This is repeated until the decanted fluid gives no cloud with a drop of 1 per cent hydrochloric acid. It is then washed with alcohol and ether and dried in a desiccator. This material is brownish, rather tough, easily soluble in warm dilute alkali, and has a tendency to gel on cooling in very dilute alkaline solution. It gives a strong, light purple biuret reaction. In doses of from 0.05 to 0.1 gm. per kilo of body weight intravenously or intraperitoneally in dogs, it produces severe intoxication which is usually lethal in young animals. The symptoms are those described as following an injection of fresh extract of pancreas.

The method of recovery of this toxic protein fraction from the autolyzed gland discloses a difference in the enzymatic activity in the auto-

lyzing pancreas of the dog, as contrasted with that of beef or pig. The product was recovered in such small quantity and there was such difficulty in obtaining dog material that attempts were made to procure a similar substance from beef and pig pancreas. While the β-nucleoprotein from these glands was found to be toxic, no poisonous material could be recovered after autolysis by the method used with dog pancreas.

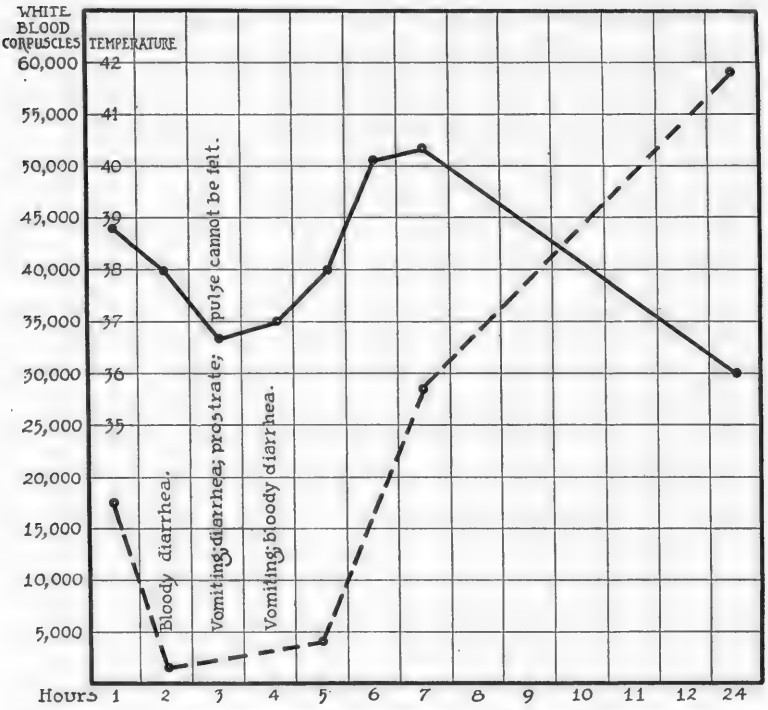
After the toxic fraction of dog pancreas is precipitated from the autolytic filtrate by acetic acid and filtered off, an abundant, white, flocculent precipitate can be thrown down from this second filtrate by the addition of a few drops of 1 per cent hydrochloric acid and an equal volume of 95 per cent alcohol. This material when purified by redissolution and reprecipitation with dilute hydrochloric acid, washed in alcohol and ether, and dried, is a granular sandy substance, which becomes a white powder when ground in a mortar. It is biuret-free, and is non-toxic for dogs in doses of 0.1 gm. per kilo. It contains an abundance of phosphorus and guanine. It gives a deep pentose color reaction and does not seem to contain adenine. In all probability it is largely composed of guanylic acid.

It is well known that guanylic acid disappears rapidly from the autolyzing pancreas of beef or pig, and our experiments have shown that the toxic protein material also cannot be recovered from these glands, whereas a poisonous protein fraction and especially guanylic acid can be obtained in relatively large amounts from autolyzing dog pancreas.

Since β-nucleoprotein is toxic and guanylic acid is not, we have assumed that the toxic material is identical or closely associated with the protein portion. Both the protein portion and guanylic acid are rapidly destroyed in beef and pig pancreas, while neither is attacked or very slowly attacked during autolysis of dog pancreas. But it is possible after autolysis, to separate the two and recover the toxic substance in greater concentration.

Petersen, Jobling, and Eggstein's[3] observations upon the serum changes during intoxication from acute hemorrhagic pancreatitis suggest that poisoning results from absorption of protein material, and it seems more probable that the protein itself which we have isolated is a toxic agent than that some other material is associated with it.

The separation by Whipple[4] of a toxic proteose from the glands in hemorrhagic pancreatitis indicates that the protein substance I have isolated is probably not the sole active agent in the production of toxic symptoms in this disease. Perhaps many different injurious



TEXT-FIG. 1. Temperature and leukocyte chart of a dog following the intravenous injection of 0.05 gm. of toxic protein of the pancreas per kilo of body weight. The solid line represents the temperature, the broken line the number of leukocytes.

substances may be absorbed from such a complex material as an autolyzing pancreas.

Text-fig. 1 summarizes an experiment in which the concentrated toxic protein was injected intravenously. Because of the small quantity of material available, pups weighing from 6 to 8 pounds

were used. The purified dry substance was dissolved in 10 cc. of very dilute sodium carbonate and injected rapidly into the jugular vein. 50 mg. per kilo of body weight were used. The animal recovered.

CONCLUSIONS.

1. A toxic constituent is present in the fresh pancreas of dogs before trypsinogen can have been activated.

2. The toxic fraction is present in the β-nucleoprotein, and therefore is thermostable.

3. It is gradually destroyed by autolysis but can be obtained in concentrated form from a gland which has autolyzed 24 hours.

4. The toxic material is probably protein in nature.

5. Intravenous or intraperitoneal injection of from 0.05 to 0.1 gm. per kilo of body weight is usually fatal and elicits in dogs symptoms comparable to those of spontaneous hemorrhagic necrosis of the pancreas.

6. Dog pancreas differs from that of beef or pig in that guanylic acid and toxic protein combined with it are slowly broken down by autolysis.

7. After autolysis of dog pancreas the toxic protein and guanylic acid may be separated and obtained in relatively pure form.

THE CYCLIC CHANGES IN THE MAMMARY GLAND UNDER NORMAL AND PATHOLOGICAL CONDITIONS.

I. The Changes in the Non-Pregnant Guinea Pig.

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INTRODUCTION.

While the literature dealing with the growth and function of the mammary gland is extensive, the mechanism determining the growth processes as well as secretion is incompletely known. Growth and function of the mammary gland are so closely interrelated with the cyclic changes of the uterus and ovaries that an understanding of the latter is a prerequisite for the former.

During the last 10 years one of the writers has brought to a conclusion the analysis of the mechanism of the mammalian, uterine, and ovarian cycle in its principal outlines. On the basis of the previous studies it is now possible to attempt a further analysis of processes of growth and secretion in the mammary gland. While the results in this and the succeeding paper clear up certain phases of the problem, it will be necessary in further studies to complete the analysis.[1]

The conclusions embodied in this and the succeeding paper are based on the examination of the mammary glands in guinea pigs. In almost all the animals examined the period of heat had been

[1] During the summer of 1915 a number of the mammary glands were prepared for microscopic examination in the Pathological Laboratory of the St. Louis University Medical School. The facilities of this laboratory were generously placed at our disposal by the authorities of the School and by the Director of the laboratory, Dr. Ralph L. Thompson. We take this opportunity to express our appreciation of the assistance accorded us.

observed, and the period of the sexual cycle of the animal was therefore known. It was confirmed by microscopic examination of the ovaries and uterus in the majority of cases; the former were invariably cut in serial sections and thus a comparison between the condition of the ovaries and uterus on the one hand, and of the mammary gland on the other, was made possible. In addition, the majority of the animals were subjected to certain experimental procedures, under ether anesthesia, at a known period of the sexual cycle, in order to determine the significance of the ovaries as a whole, of the corpora lutea, of the uterus, of the deciduomata, with and without pregnancy, and of lutein injection for the condition of the mammary gland.

In order to avoid the necessity of describing in detail in each case the state of the mammary gland as revealed on microscopic examination, we characterized the mammary gland as proliferating, as resting, or as intermediate. We disregarded almost entirely the amount of gland tissue which we found at the time of examination. The latter depended not so much on the stage of the cycle in the mammary gland and on the experimental interferences as apparently on more or less accidental factors, particularly on the time which had elapsed since the last pregnancy preceding the time of examination. We shall describe briefly the different types of mammary glands.

Proliferating Mammary Gland.—The acini and large ducts are of variable size, sometimes very large, and even cystically dilated. The epithelium is high cuboidal or almost cylindrical; in some instances it is only medium cuboidal. The epithelial nuclei are vesicular. There are mitoses present in the acini, sometimes in large numbers, in other glands they are less frequent; in some cases they are only rarely found. The amount of colloid-like material within the acini or ducts varies in different glands. There may be papilla-like protrusions into the large ducts. The connective tissue between the acini is scant, more or less cellular, with large, sometimes with smaller vesicular nuclei. Lymphocytes and polynuclear leukocytes are usually found in the stroma. There is usually much fluid present in the stroma. In some glands mitoses are found in the fibroblasts of the stroma; in others they are absent. The amount and character of the stroma are not identical in all glands;

there may occasionally be some stronger development of fibrous tissue, especially in certain parts of the gland; the gland may be proliferating only in parts.

Resting Mammary Gland.—We usually find small acini, occasionally cystically dilated acini, lined by low cuboidal or flat epithelium, with dark staining, condensed or small vesicular nuclei. The gland ducts have likewise low epithelium. Desquamated epithelial cells may be present in the lumen of the acini. Colloid-like material may be present or absent. Mitotic proliferation is entirely lacking. The connective tissue between the acini or around the ducts is usually densely fibrous and often present in a relatively large amount. The nuclei in the stroma are small and compressed. Mitoses are absent. Polynuclear leukocytes and lymphocytes are usually not seen.

Intermediate Mammary Gland.—While the types of proliferative and resting mammary gland are fairly sharply defined, this is not so in the same degree in the third type, the intermediate mammary gland. Here mitoses are always absent, but the structure of the gland and stroma does not approach the inactivity of the resting gland, but approaches more or less that of the proliferating gland. The gland epithelium is higher than in the resting gland, and the stroma, in parts or throughout, is more cellular than in the resting gland; the nuclei are more vesicular, and the quantity of dense hyaline connective tissue, throughout the gland or at certain places, is much diminished. Lymphocytes and leukocytes are often present, but the characteristic feature of the proliferating gland, the mitoses, are absent. The intermediate gland may otherwise bear a greater resemblance to the proliferating or to the resting gland and we may therefore make further subdivisions, designating some glands as intermediate to resting, others as better than intermediate.

Normal Cycle of the Mammary Gland in the Non-Pregnant Animal. Period from Time of Heat to 5 Days after Ovulation.

In the guinea pig the stage of heat can be recognized by examination of the ovaries and uterus. A detailed description of these changes has been previously given by one of us.[2] In the observations

[2] Loeb, L., *J. Morphol.*, 1911, xxii, 37; *Virchows Arch. path. Anat.*, 1911, ccvi, 278; *Biol. Bull.*, 1914, xxvii, 1.

reported in this communication the diagnosis of the period of heat is based on the state of these organs as shown by microscopical examination. In some cases the objective signs of heat were not yet completely present, but even in these cases, knowing that the interval since the preceding ovulation corresponds to a full period of the sexual cycle, and finding those changes in the uterus and ovaries which approach the characteristics of heat, we were in a position to diagnose an impending heat.

Mammary Gland near the Stage of Heat or during Heat.—We examined the mammary gland in seven guinea pigs which were either in heat or near heat. In all of them we found proliferating glands; in no case was the gland in a resting or intermediate condition. In the guinea pig the period of heat is usually followed by ovulation. The latter generally occurs 6 to 12 hours after copulation, but in the guinea pig a preceding copulation is not, as it is in some animals, a prerequisite for ovulation. The time immediately preceding or following ovulation can be recognized by a microscopic examination of the ovaries, which shows greatly enlarged mature follicles either near the stage of rupture or directly following rupture.

Mammary Gland near Ovulation or within 24 Hours after Ovulation.—We examined the mammary glands of eight guinea pigs which were either at the point of ovulation or in which ovulation had taken place within the past 24 hours. In five of these animals the glands were proliferating. In three of the latter animals incisions had previously been made into the uterus and deciduomata had developed, which at the time of examination had become necrotic. Ovulation, however, had not been delayed by the presence of necrotic deciduomata. In two other cases the gland showed an intermediate character, and in one case we found a gland in the resting state. In the latter instance, incisions had been made in the uterus, and deciduomata had developed and caused a delay in ovulation. It was $26\frac{1}{2}$ days after the previous ovulation before a new ovulation took place; the deciduomata were found necrotic soon after ovulation. In this case of delayed ovulation a resting gland was found in spite of the period of the sexual cycle at which the examination had taken place. Here apparently the delay in ovulation caused by the pres-

ence of deciduomata was responsible for the lack of progressive changes in the mammary gland.

Mammary Gland from 24 to 72 Hours after Ovulation.—We examined sixteen guinea pigs in this period of the sexual cycle. In seven we found a proliferating gland. In one of these a necrotic deciduoma was present, but ovulation had taken place at the proper time. In seven animals the glands were intermediate; in two of these we found necrotic deciduomata but there had not been any delay in ovulation. In two animals the glands were resting, or slightly better. In both of these necrotic deciduomata were present. In one of the animals ovulation had been delayed, and in the second it had taken place at the proper time. We found, therefore, a proliferating gland in not quite one-half of the animals, but a resting gland only in a small minority.

Mammary Gland from 72 Hours to the End of the 5th Day after Ovulation.—At this stage of the sexual cycle we find six proliferating glands. No incisions in the uterus had been made and no deciduomata had, therefore, developed. In five animals the glands were intermediate; ovulation had taken place at the proper time in three, and the remaining two died a few days after ovulation. In no case had incisions been made into the uterus. In five animals the glands were somewhat better than resting, but not quite intermediate. At this period a little more than one-third of the glands were proliferating and almost one-third were slightly better than resting.

If we summarize the results, we find that during a period of the sexual cycle including the time preceding heat until 5 days after ovulation, there is a gradual decrease in the activity of the mammary glands. At the time of heat all the glands proliferate, and from 3 to 5 days after ovulation, little more than one-third are proliferating and almost one-third are near the resting state. There is a maximum at the period of heat and from then on there is a gradual decline to the end of this period. In some instances in which a delay of ovulation had been experimentally produced, the mammary gland seemed to show less activity during this period than in the animals in which ovulation took place at the usual time. Our observations, however, merely suggest the latter conclusion.

Period from 5 to 15 Days after Ovulation.

Mammary Gland from the Beginning of the 6th to the End of the 12th Day after Ovulation.—Twenty-nine animals in this period of the sexual cycle were examined. There was in addition one guinea pig which presumably belonged to this stage. Of these, fifteen or sixteen animals showed an intermediate, and fourteen an approximately resting condition of the mammary gland; the majority of the latter were slightly better than resting. In no case was a necrotic deciduoma present in the uterus. A proliferating gland was not found during this period. Almost half the glands were, therefore, in a resting, and the rest in an intermediate condition.

Mammary Gland from the Beginning of the 13th to the End of the 15th Day after Ovulation.—Four animals were examined in this stage of the sexual cycle. One showed a proliferating mammary gland. In this case a foreign body had been introduced into the uterus $2\frac{1}{2}$ days after heat; 6 days after heat the foreign body was removed and incisions were made into the uterus. Three good corpora lutea were found in the ovaries. In three other animals the mammary glands were resting or slightly better than resting. In the first of these a good corpus luteum and mature follicles were present. In the second we found good corpora lutea as well as a small deciduoma and mature follicles. In the third we found partly preserved, partly necrotic deciduomatous tissue, and lutein injections had been made.

In this stage of the sexual cycle the mammary gland is, therefore, on the whole inactive. We may furthermore conclude that at this stage of the cycle neither mature follicles nor well preserved corpora lutea nor the presence of a small deciduoma are usually able to stimulate the mammary gland to activity, although in one case where good corpora lutea were present, proliferation had taken place.

If we summarize the findings during this whole period, we may conclude that the time from the 6th to the 15th day after ovulation represents the lowest level in the activity curve of the mammary gland, although good corpora lutea are usually present and mature follicles may appear in the second half of this period.

Period from 16 to 27 Days after Ovulation.

The normal sexual cycle in the guinea pig varies between 15 and 19 days. This series includes, therefore, not only guinea pigs in the last stage of the sexual cycle, but also animals in which normal ovulation had been delayed.

Mammary Gland from the Beginning of the 16th to the End of the 19th Day.—In this period we find five proliferating glands. In three of these cases the uterus had been extirpated and the corpora lutea were well preserved. In the two other cases vacuolar corpora lutea were present. Mature follicles were noted in two of the five cases. In four cases the mammary glands were resting. In one of these animals the ovaries were hypotypical; in three, good deciduomata were present. In two of the latter the ovaries had not been examined; and in the third the corpora lutea showed beginning vacuolization and mature follicles were present. In two animals the glands were intermediate. In one of these the structure of the gland approached that of a proliferating gland, but mitoses were not definitely observed. The condition of the uterus and ovaries indicated that this animal was near the period of heat. In the second case vacuolar corpora lutea and reactive follicles were present. But there was not yet any other indication of approaching heat. In this case the mammary gland at one place approached the proliferating stage. We find then that at this stage of the sexual cycle approximately one-half the animals had a proliferating gland. Proliferation occurs at this period in cases in which, while a new ovulation is not approaching, the corpora lutea are well preserved (probably as the result of the extirpation of the uterus).

In a few other cases in which apparently the stage of heat is approaching, we find good intermediate or even proliferating glands. Apparently the same factors which prepare uterus and ovaries for heat, exert also some effect on the mammary gland. Notwithstanding the presence of good deciduomata and of mature follicles, the gland may still be resting in certain other cases. In one the ovaries were hypotypical, a condition which explains the inactivity of the mammary gland which is accompanied by an inactive state of the uterus.

Mammary Gland from the Beginning of the 20th to the 27th Day.— This period does not occur under normal conditions. Under normal conditions a new ovulation usually precedes this period. But if through incisions in the uterus, made 4 to 8 days after ovulation, deciduomata are experimentally produced, a new ovulation is often delayed, and the sexual period prolonged. The mammary glands of twenty-four animals were examined during this period; of these fifteen were resting glands, five were proliferating, and four were intermediate. The five proliferating glands were found in animals 21 to 25 days after previous ovulation. In the first animal the thyroid had been entirely or almost entirely extirpated. Corpora lutea as well as mature follicles were present. In the second incisions had been made in the uterus; the developing deciduomata had become necrotic, and the corpora lutea showed vacuolization. In this case there was little proliferation present. Proliferation in this case may have started at an earlier period, while deciduomata and corpora lutea were still in good condition. In the third animal incisions had been made in the uterus, infection of the uterus took place, and although the animal was sick, the corpora lutea were well preserved. In the fourth instance the uterus had been completely extirpated. Deciduomata were not present, but as usual under these conditions the corpora lutea were preserved and there were mature follicles present. In the fifth the corpora lutea were necrotic, but the state of the mature follicles suggested that the animal was near ovulation. In three of the guinea pigs, therefore, well preserved corpora lutea were present. Sickness of the animal due to infection of the uterus does not necessarily prevent proliferation of the mammary gland. In one of the remaining animals impending ovulation was probably responsible for the proliferation. The four animals in which one intermediate gland was found had all ovulated 21 to 23 days previously. In all these guinea pigs the corpora lutea were vacuolar, and there were in addition necrotic deciduomata. In two of the animals mature follicles were found.

The fifteen guinea pigs with resting glands or with glands slightly better than resting had ovulated during a period varying between 21 and 27 days preceding the examination. Of these the majority, namely eleven, showed degenerated corpora lutea; of the remaining

four two had good corpora lutea, in two others the corpora lutea were partly preserved; these four animals, however, had deciduomata which were entirely or partly necrotic. Of those animals which had vacuolar, retrogressing corpora lutea, five showed in addition necrotic deciduomata. Mature follicles were found in the ovaries of two of the guinea pigs, without, however, showing any indications that an ovulation was imminent.

We see then that in those animals in which there was a resting mammary gland degenerating corpora lutea were present in the majority of cases. In a few instances in which the corpora lutea were partly preserved, deciduomata were necrotic, while in two animals mature follicles were found. In none of these animals was there any indication of an imminent heat. It is also of interest to note that all the animals in which more than 23 days had elapsed since the previous ovulation, in which, therefore, the delay in ovulation was especially great, the mammary gland was in a more or less resting stage. These facts are of interest in considering the succeeding set of animals.

Mammary Gland in Guinea Pigs in Which Living Deciduomata and Well Preserved Corpora Lutea Are Present.

It was advisable to make a separate group of these animals in order to determine whether the presence of experimentally produced deciduomata without the accompaniment of embryonic structures, together with the presence of good corpora lutea, affect the mammary gland. It was especially of interest to compare these animals with others of the corresponding period of the sexual cycle in which pregnancy was found.

We examined the mammary gland in twenty-five animals belonging to this group. Twenty-one of the animals have not been included in the various groups of the normal sexual cycle; in these ovulation had occurred later than 16 days previous to examination, while the four animals in which ovulation had occurred from 12 to 16 days previously have been referred to above.

Mammary Gland from the 9th to the End of the 13th Day of the Sexual Cycle.—Seven animals belong to this class. One had a proliferating

gland; it has been mentioned among the animals in the normal sexual cycle. Two animals, both of which were sick, had a resting gland. Of four in which the gland was intermediate, one showed mature follicles. We see, therefore, that at this stage of the cycle the glands as a rule show no proliferation notwithstanding the presence of good deciduomata and well developed corpora lutea.

Mammary Gland from the Beginning of the 14th to the End of the 17th Day.—In three animals the mammary gland was proliferating. In two the corpora lutea showed a slight peripheral vacuolization, and in the third the corpus luteum was good throughout. In two of these three animals mature follicles were present. Three animals had an intermediate gland. In one of these the corpora lutea showed the beginning of vacuolization. In another the ovaries had not been examined, but there is little doubt that the corpora lutea were at this period still fairly well preserved. Mature follicles were also present in one animal. One-half the guinea pigs, therefore, had a proliferating, while the remainder showed an intermediate gland.

Mammary Gland from the Beginning of the 18th to the End of the 20th Day.—All three animals showed a proliferating gland. The corpora lutea were well preserved, and mature follicles were present in all of them.

If we combine the second and third groups, we have nine guinea pigs of which two-thirds show a proliferating, and the remaining one-third an intermediate gland. If we compare this result with the condition in animals of the normal cycle without the presence of good deciduomata, and in approximately the corresponding period of the sexual cycle, we find much greater activity in the mammary gland of animals in which good deciduomata are present. Without good deciduomata during an approximately corresponding period we find the majority of glands in a resting condition and only a little more than one-third proliferating.

Mammary Gland from the Beginning of the 21st to the End of the 23rd Day.—Nine animals were examined at this period. In six with good deciduomata and corpora lutea which were well preserved or showed just beginning peripheral vacuolization, the mammary gland was proliferating. In all but one of these animals mature follicles were present in the ovaries. In one animal with good corpora lutea

and with deciduomata that showed slight degeneration, it was doubtful whether the mammary gland was proliferating or intermediate. In this case also mature follicles were present. In the last two guinea pigs in which there were also good deciduomata and good corpora lutea present, the mammary gland was in one case intermediate, in the other not quite intermediate.

Mammary Gland from the Beginning of the 24th to the Beginning of the 27th Day.—In all these four animals good deciduomata and corpora lutea that were well preserved or showed merely beginning peripheral vacuolization were present. All had mature follicles. Three showed a proliferating gland, one a gland that was better than intermediate.

From the 21st to the 27th day the mammary glands of thirteen animals were examined. Nine of these were definitely proliferating, one perhaps proliferating, and the remaining three were intermediate. The majority were, therefore, proliferating; none were resting in this group. If we compare these glands with the mammary glands of animals of the corresponding period, in which the deciduomata were mostly necrotic, we find a great difference. In the latter the majority were in a resting condition. It is probable that both preservation of corpora lutea as well as deciduomata contributed to this result. In the former group of corresponding age in several instances we found a good corpus luteum without deciduomata, combined with a proliferating gland. However, in cases in which necrotic deciduomata were present, the mammary gland was usually resting, while in this group with good deciduomata the mammary gland was usually proliferating. We may then conclude that in cases in which the sexual cycle is artificially prolonged, well preserved deciduomata are usually associated with proliferating glands, while degenerating deciduomata which are usually combined with vacuolar corpora lutea, are associated with resting glands. Good corpora lutea and good deciduomata may, therefore, at this period of the sexual cycle cause proliferation of the mammary gland without the presence of pregnancy.

Mature follicles, such as are found even in cases in which vacuolar corpora lutea and necrotic deciduomata are present, are not able to cause proliferation in the mammary gland. Even at an earlier date, in the period from the 16th day on, the presence of good cor-

pora lutea combined with good deciduomata in the majority of cases produced proliferation of the mammary gland.

The period from the 9th to the 14th day of the sexual cycle belongs to the refractory period of the sexual cycle. At this time even the presence of good corpora lutea combined with well preserved deciduomata could only in one case produce proliferation; but in the period from the 14th to the end of the 24th day of the sexual period, there were fifteen or sixteen proliferating glands and six which were intermediate. It is probable that the deciduomata contribute to the effect of the corpus luteum. In three out of four cases in which good corpora lutea were present, but the deciduomata necrotic, the mammary gland was resting, while in the fourth case it was proliferating notwithstanding the necrotic condition of the deciduoma and a somewhat vacuolar state of the corpus luteum.

We see then that a definite cycle of the mammary gland corresponds to the cycle in the ovary and uterus. In the first period we find proliferation of the mammary gland, which gradually diminishes as the sexual cycle progresses, then comes a resting period at a time when the corpus luteum is well developed and functioning, to be followed again by beginning proliferation at a period when the new sexual cycle in the ovary and uterus is approaching. If we prevent the onset of the new sexual cycle and artificially prolong the old cycle, then we find that proliferation of the mammary gland is usually associated with the presence of good deciduomata and corpora lutea, while in the presence of necrotic deciduomata, which are often but not always accompanied by vacuolar corpora lutea, the mammary gland usually remains resting.

Effect on the Mammary Gland of Extirpation of the Ovaries.

In nineteen guinea pigs both ovaries were extirpated,—in the majority of instances 5 to 7 days, in some, however, as early as 3 days after ovulation. In all but two incisions were made in the uterus at the time of castration. The development of deciduomata was interfered with; they either did not develop at all or remained small and had become necrotic, if the examination took place 20 days after ovulation or later. In the two animals in which no incisions had

been made in the uterus, the examination was made approximately 18 days after ovulation; the mammary gland was resting or intermediate. In one case in which incisions in the uterus had been made, on examination 14 days after ovulation, the mammary gland proved to be resting or slightly better than resting. In this guinea pig little deciduomatous tissue had been produced.

In another in which the examination took place 9 days after ovulation the gland was almost resting. In a third animal 14 to 15 days after ovulation a small deciduoma had developed which had become entirely necrotic and showed many hemorrhagic areas. The mammary gland was resting to intermediate. In a fourth and fifth instance at the examination $9\frac{1}{2}$ to $10\frac{1}{2}$ days after ovulation, the mammary gland was likewise resting to intermediate. In these five guinea pigs the mammary glands were, therefore, examined during the negative period. Proliferation could not be expected at that period even with the presence of normally functioning ovaries. In the other twelve cases examination took place 20 to 22 days after ovulation. In almost all, deciduomata had developed which had become necrotic. The mammary gland varied in these cases between the resting and intermediate condition.

As we have shown, in the presence of good corpora lutea or of a combination of good corpora lutea and good deciduomata, proliferation of the mammary gland is frequent between the 18th and 22nd days after ovulation. After extirpation of the ovaries, however, proliferation was absent during this period. We may therefore conclude that extirpation of the ovaries prevents proliferation of the mammary gland. After castration those influences emanating from the uterus and ovaries which, under certain conditions, cause a proliferation of the mammary gland, are absent. It may be that the same condition which leads to the early necrosis of the deciduomata after castration also prevents proliferation of the mammary gland. In addition, the absence of good deciduomata or the presence of necrotic deciduomata may in such cases have an additional unfavorable influence on the mammary gland.

Mammary Gland in Animals with Hypotypical Ovaries.

Loeb has formerly described a condition of the ovaries in which the follicles do not reach full size.[3] Atresia sets in at a stage when the follicles are as yet of medium size or even smaller. Such ovaries he designated as hypotypical. It seems that in almost all these cases corpora lutea are either degenerating or absent. We observed ten cases of this kind in which the condition of the mammary gland was ascertained. While in the majority of the animals there can be no doubt about the character of the ovaries, it is possible that in an exceptional case an accidental transitory condition was included in this series. In all these guinea pigs the mammary gland was resting or only slightly better.

In one of the animals the ovaries were examined 6 days after ovulation. The corpora lutea had been extirpated 4 days previously. In the second case incisions had been made in the uterus and only the remnants of degenerated corpora lutea were visible. The third guinea pig died 13 days after heat; the animal was in a state of inanition; the corpora lutea showed beginning vacuolization. In the fourth animal a few small pieces had been cut out from the ovaries 5 days after ovulation; 22 days after ovulation retrogressing vacuolar corpora lutea were found. In the fifth the thyroids had been (perhaps incompletely) removed $4\frac{1}{2}$ days after ovulation; $23\frac{1}{2}$ days after ovulation vacuolar corpora lutea were found. In the sixth instance, 26 days after thyroidectomy, very much degenerated remnants of corpora lutea were found. In the seventh guinea pig the thyroids had been extirpated $5\frac{1}{2}$ days after ovulation. On examination 23 days later degenerating corpora lutea were found; no new ovulation had taken place. In both the sixth and seventh cases the animals had been sick before examination of the ovaries. In the eighth case small pieces had been excised from the ovaries almost 4 days after ovulation. 10 days later the guinea pig died. A large, well preserved, corpus luteum was present. In the ninth animal blood had been withdrawn on two occasions, and 3 days after the last bleeding the animal was killed. Only the remnants of corpora lutea were present. In the last case an incomplete extirpation of corpora lutea

[3] Loeb, *Zentr. Physiol.*, 1911–12, xxv, 342.

had been done 7 days after ovulation; 10 days later the examination showed the presence of vacuolar, small corpora lutea. A new ovulation had not taken place in many of these cases.

We find, then, that hypotypical ovaries are combined with an inactive state of the mammary gland and that guinea pigs with hypotypical ovaries behave like castrated guinea pigs.

Effect of Extirpation of the Corpora Lutea on the Mammary Gland.

We stated above that in cases in which the ovaries were hypotypical the corpora lutea were usually absent or degenerating, and that in these cases the mammary gland was inactive. A similar condition of the mammary gland is observed in cases in which at an early stage of the sexual cycle the corpora lutea have been extirpated without a new ovulation taking place. As one of us has shown previously, extirpation of the corpora lutea leads in the majority of cases to a hastening of the new ovulation which marks the onset of a new sexual cycle.[4]

We examined the mammary gland in thirty-three guinea pigs n which the corpora lutea had been completely extirpated. We may divide this set into two groups: in the first one, comprising thirteen animals, a new ovulation had not yet taken place at the time of examination; one of these animals has already been mentioned among the guinea pigs in which the ovaries were hypotypical. In the second group, comprising twenty animals, a new ovulation had occurred at the time of examination.

(*a*) In the first group we found a resting mammary gland in six animals which were examined at a period from 4 to 17 days after ovulation. In these animals the corpora lutea had been absent from 2 to 11 days. In one of these cases incisions had been made into the uterus 6 days after ovulation; on examination 10 days later, small living deciduomata were found. The examination in these six guinea pigs was made at a time, when, during the normal cycle, no proliferation usually occurs in the mammary gland.

In seven instances the mammary gland was intermediate. In four of the animals the examination took place at an early period of the

[4] Loeb, *Deutsch. med. Woch.*, 1911, xxxvii, 17.

sexual cycle; namely, from 5 to 9 days after the ovulation which preceded the extirpation of the corpora lutea. In these four the corpora lutea had been absent from 3 to 7 days. In the remaining three cases incisions had been made into the uterus, in two 6 days, and in one $3\frac{1}{2}$ days after ovulation. In two of the animals no deciduomata developed owing to the absence of the corpora lutea, and in the third they had become necrotic at the time of examination. Examination was made in these cases $17\frac{1}{2}$ days after the ovulation which preceded the extirpation of the corpora lutea. The corpora lutea had been absent 13 or 14 days. In two of these guinea pigs mature follicles were present and one of them was in heat. Notwithstanding the presence of heat, this animal, in which deciduomata had not developed in the cut uterus, did not show a proliferating gland. In the third animal, in which we were only able to examine one ovary, it is doubtful whether a new ovulation had not taken place. We find, therefore, after extirpation of the corpora lutea an intermediate gland either in animals with mature follicles or in animals examined during the first half of the sexual cycle, the latter class representing cases in which the period of absence of corpora lutea had been relatively short. It is probable that considering the occasional presence of proliferation in the mammary gland during the corresponding period of the normal cycle and its complete absence after extirpation of the corpora lutea, that the absence of corpora lutea is partly responsible for the non-proliferating condition in the mammary gland. On the other hand, we have seen that if heat or ovulation occurs, proliferation of the mammary gland sets in during the normal cycle notwithstanding the absence of good corpora lutea. This latter conclusion is confirmed by experiments in which after extirpation of the corpora lutea, a premature new ovulation took place.

(*b*) The second set comprises twenty animals in which after extirpation of the corpora lutea, a new ovulation was imminent or had taken place. As stated above, after complete extirpation of the corpora lutea the new ovulation is generally accelerated.

Two animals were found near or during the stage of heat. In one of these in which the uterus had not been cut, the mammary gland was proliferating. In this case the preceding heat had occurred a little over 12 days previously, and the corpora lutea had been

absent 10 days. The second animal has already been mentioned under (*a*). In this case incisions had been made in the uterus, but no deciduomata developed. Examination was made 17½ days after ovulation; corpora lutea had been absent for 14 days. The mammary gland was almost intermediate. In three cases the animals were near ovulation or ovulation had taken place within the preceding 24 hours. No incisions had been made into the uterus. Corpora lutea had been absent in these animals for 8, 9, and 14 days, respectively, and extirpation of the corpora lutea had taken place from 2 to 3 days following ovulation. In two of the animals the mammary gland was proliferating, in the third its structure was almost that of a proliferating gland, but mitoses were not found. In three animals examination took place from 1 to 3 days after ovulation. Here the second ovulation took place from 11 to 15 days after the first one, and corpora lutea had been absent during a period varying from 7 to 9 days. In all these animals the mammary gland was proliferating.

In six guinea pigs in which the ovulation following extirpation of the corpora lutea had taken place 3 to 5 days, and in seven other guinea pigs in which the second ovulation had taken place 5½ to 6 days previous to examination, the mammary gland was found in an approximately intermediate condition. In most of these animals extirpation of the corpora lutea, which was complete in each animal, as microscopic examination showed, had been carried out 2 to 3 days after the first observed ovulation. Examination took place 11 to 15 days after extirpation of the corpora lutea and the ovulation following extirpation of the corpora lutea was accelerated in all cases. In a number of these cases incisions had been made in the uterus, but deciduomata did not usually develop. Only in animals in which the corpora lutea had been extirpated relatively late, namely, 6½ days after ovulation, did deciduomata develop; in one of these they had become necrotic, in the second small, good deciduomata were visible at the time of examination.

These experiments confirm the conclusion at which we arrived through our observations of the normal cycle; namely, that in the guinea pig the stage of heat and ovulation and the period immediately succeeding it are accompanied by a proliferation of the mammary gland, while the later period, coincident with the development

of the corpus luteum, is not accompanied by a proliferation. We learn, furthermore, than an absence of corpora lutea during a certain period preceding heat and ovulation does not only not prevent subsequent proliferation of the gland, but is a prerequisite for this proliferation, inasmuch as without a preceding degeneration or extirpation of all the corpora lutea no new heat or ovulation can take place. This conclusion is in accordance with the fact previously demonstrated by one of us that corpora lutea, probably through chemical influence, inhibit heat and ovulation.[4] In a similar manner the corpora lutea inhibit that proliferation of the mammary gland which is characteristic of the first period of the sexual cycle. It is probable that this particular influence of the corpora lutea on the mammary gland is not a direct, but an indirect one,. the direct action being exerted on the sexual cycle of ovaries and uterus, which act on the mammary gland.

While extirpation of the ovaries prevents the onset of a new sexual cycle and therefore the concomitant proliferation of the mammary gland, extirpation of the corpora lutea hastens both of these processes. Another constituent, namely, in all probability the follicular apparatus or part of it, is responsible for the occurrence of heat, ovulation, and the proliferation of the mammary gland in the early period of the sexual cycle.

Effect of Extirpation of the Uterus on the Mammary Gland.

Loeb has previously observed that extirpation of the uterus seems to have a preserving influence on the corpora lutea.[5] It is therefore of interest to contrast specially this group of experiments with the preceding group in which the corpora lutea were absent. In four animals the mammary gland had been examined after an extirpation of the uterus, which had been carried out at an early period of the sexual cycle. Examination took place in one case 17, in two cases 18, and in the fourth case 23 days after the preceding ovulation. In two small deciduomata were present at the lower stumps of the uterus. Excision of the uterus had, therefore, not been complete.

[5] It is intended to investigate further this relationship between extirpation of the uterus and the life of the corpora lutea.

In the remaining cases deciduomata were not found. In all four instances the corpora lutea were well preserved and the mammary gland was proliferating. It is probable that the proliferation of the mammary gland and the preservation of the corpora lutea in these cases stand in a causal relation to each other.

SUMMARY.

1. A definite cycle exists in the mammary gland of the non-pregnant guinea pig which corresponds to the cycle in the ovary and uterus. This cycle can be presented through a curve in which the ordinates represent the degree of activity of the gland in a series of animals, and the abscissæ the time since ovulation (period of sexual cycle). The curve passes through a first maximum at the time of heat and ovulation and gradually falls. The minimum is reached on the 6th day and continues until the 15th day after ovulation. Next begins the period when a new ovulation is imminent and the number of the proliferating glands again increases. We see then that during the normal cycle the presence of well preserved, functioning corpora lutea does not lead to proliferation, neither do mature follicles have such an effect. On the other hand, the absence or degeneration of the corpora lutea is required to insure the proliferation of the mammary gland in the first period of the sexual cycle.

If the sexual period is experimentally prolonged, we find in some instances proliferation, while in others it is absent. As far as we can determine at the present time, two factors seem to favor proliferation of the mammary gland under these conditions: (1) the presence of well preserved corpora lutea, particularly if they are associated with well preserved experimentally produced deciduomata, and (2) the imminence of a new period of heat. The connection between good corpora lutea and good deciduomata and the presence of proliferating mammary glands at this stage of the sexual cycle is, however, not absolute. There are cases in which a proliferating gland is associated with some degeneration of the corpus luteum. Or on the other hand a well preserved corpus luteum is associated with a non-proliferating gland. In some of the latter cases the simultaneous presence of a necrotic deciduoma may perhaps explain the lack of proliferation in the mammary gland. However, in the majority of

cases we found the presence of good corpora lutea and good deciduomata associated with a proliferating mammary gland. Whether a living corpus luteum as such is able to produce proliferation of the gland is as yet doubtful.

2. Extirpation of the ovaries prevents not only the proliferation of the mammary gland associated with the first stage of the sexual cycle, the condition of heat and ovulation no longer taking place in castrated animals, but in all probability also inhibits the proliferation of the mammary gland which occurs under certain conditions towards the end of the sexual cycle, or in instances of experimentally prolonged sexual cycle in which well preserved corpora lutea and deciduomata are present.

3. In animals in which the ovaries were hypotypical, the mammary glands were in an inactive condition. The presence of hypotypical ovaries has the same influence on the mammary gland as castration. In the majority, but not in all of these cases well preserved corpora lutea were absent.

4. Complete extirpation of the corpora lutea seems directly or indirectly to prevent the secondary proliferation of the mammary gland, which occurs during the latter part of the sexual cycle or during an experimentally prolonged cycle, in cases in which the extirpation is not followed at once by a new ovulation. This conclusion we consider, however, merely as suggested, not yet as definitely established through our results. On the other hand, the primary proliferation of the mammary gland, during the first stage of the sexual cycle, as well as ovulation and the objective signs of heat, is accelerated through complete extirpation of the corpora lutea. Thus the effect of extirpation of the corpora lutea differs from the effect of castration, in that after the latter neither a new heat nor the primary proliferation of the mammary gland occurs. As one of the authors has pointed out previously, the absence of functioning corpora lutea and the presence of either well developed ovarian follicles or of mature follicles are necessary for the occurrence of heat and ovulation. The same conditions are prerequisites for the primary proliferation of the mammary gland.

5. In cases in which the whole or almost the whole uterus had been extirpated, the corpora lutea were well preserved and the mammary gland was proliferating.

THE CYCLIC CHANGES IN THE MAMMARY GLAND UNDER NORMAL AND PATHOLOGICAL CONDITIONS.

II. The Changes in the Pregnant Guinea Pig, the Effect of Lutein Injections, and the Correlation between the Cycle of the Uterus and Ovaries and the Cycle of the Mammary Gland.

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In a previous paper[1] we analyzed the cyclic changes in the mammary gland in the non-pregnant guinea pig. In this communication we shall report on our experimental studies of the mammary gland in guinea pigs during or following the period of pregnancy. We shall furthermore report on the effect of injections of lutein on the mammary gland, and in conclusion shall briefly discuss the relation between the sexual cyclic changes in the ovaries and uterus on the one hand and in the mammary gland on the other.

Mammary Gland during Different Periods of Pregnancy.

In twenty-six guinea pigs the mammary gland was examined during different periods of pregnancy.[2] Included in this series is one case of experimentally produced extra-uterine pregnancy, of which a more detailed description has been previously given by one of us[3] from another point of view. This series also included a case

[1] Loeb, L., and Hesselberg, C., *J. Exp. Med.*, 1916, xxv, 285.

[2] When the intervals indicated in the protocols had elapsed the guinea pigs were allowed to die under ether anesthesia, and the tissues taken out at operation were placed immediately into Zenker's fluid.

[3] Loeb, L., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 103; *Biol. Bull.*, 1915, xxviii, 59.

in which pregnancy was present in one horn of the uterus, while in the other horn there was a beginning abortion, and a third case in which it is doubtful whether pregnancy or merely an experimental deciduoma was present. In the remaining twenty-two cases the animals had been used for certain experiments which, however, did not interfere with the progress of pregnancy, and we have every reason to assume that they did not influence the condition of the mammary gland.

In five instances the mammary gland was examined within the first 15 days after copulation. In one of these, however, the period of pregnancy may perhaps have been slightly farther advanced. In these five cases the mammary gland was found in the following condition: In one animal 6 days and in another about 11 days after copulation, the mammary gland was intermediate. In three cases the pregnancy was approximately 12 to 15 days old; in two of these cases the mammary gland was proliferating; in the third it was intermediate.

In seven guinea pigs the mammary gland was examined during a period from the beginning of the 16th to the end of the 19th day. In three of these animals the gland was proliferating, in four it was intermediate. In one of the latter cases, however, it was doubtful whether in addition to the deciduomata pregnancy was present.

In three animals the mammary gland was examined between the beginning of the 20th and the end of the 23rd day; it was found to be intermediate. There was in addition one case in which pregnancy was present in one horn and abortion was under way in the other horn 22 days after copulation. In this case the gland was proliferating.

In nine animals the pregnancy was in a further advanced stage, at a period later than 24 days after copulation; in all these guinea pigs the mammary gland was proliferating.

We see then that proliferation of the mammary gland during pregnancy becomes regular only after the 24th day, that it was absent in two cases on the 6th and 10th days; between the 12th and 24th days the gland was proliferating in five and intermediate in eight cases. In the majority, therefore, the gland is not proliferating in the early period of pregnancy.

If we compare the condition of the mammary gland during the first period of pregnancy with the corresponding period of the normal cycle not accompanied by copulation and pregnancy, we find the majority of glands in both non-proliferating. There were, however, during the normal cycle of the non-pregnant animal a considerable number of non-proliferating glands in a resting state, while during early pregnancy the non-proliferating glands were all intermediate. In this respect the mammary gland during this period of pregnancy resembles the mammary gland of non-pregnant animals of the same period in which good corpora lutea and good deciduomata were present. In this class of animals also the non-proliferative glands were not resting, but intermediate; the proportion of proliferative glands in the latter class was, however, even greater than during early pregnancy—a difference perhaps without significance and due to accident. It is probable that pregnancy as well as the presence of good deciduomata and corpora lutea improve the condition of the mammary gland even in those cases in which the stimulating effect of these conditions is not sufficiently strong to lead to proliferation. On the whole, however, in the guinea pig, pregnancy does not induce proliferation of the mammary gland to a much higher degree than the factors active during the latter part of the normal sexual cycle unaccompanied by pregnancy, and proliferation of the mammary gland during pregnancy becomes regular only at a period of time which exceeds the duration of the normal sexual cycle unaccompanied by pregnancy. In this group we may include an animal in which extra-uterine pregnancy had been produced experimentally. In this case an incision had been made into the uterus $2\frac{1}{2}$ days after ovulation; the examination took place 18 days after the first ovulation. The condition of the ovaries indicated that about 3 days previous to the time of examination a new ovulation had taken place. The mammary gland was proliferating,—a finding not unexpected at this stage of the sexual cycle.

Mammary Gland in the Period Following Pregnancy.

In five animals the mammary gland was examined at various periods after the completion of pregnancy.

Guinea Pig 1.—About 12 to 24 hours after delivery. In the ovaries recently ruptured follicles with the beginning of corpora lutea are visible. The mammary gland is large and consists of many lobules with numerous acini. The acini are of larger size than in the glands described above. The epithelium is high cuboidal almost throughout; in the lumen of the acini there is a finely granular precipitate, representing the secretion of the gland. Some acini are distended with secretion; here the gland cells are flat, evidently as the result of pressure. Occasionally there are small vacuoles in that part of the cell which adjoins the lumen. In a number of acini there are within the lumen yellow bodies, round or of variable shape and size, containing small pigment granules. The nuclei of the gland cells are usually large and vesicular. A few mitoses occur in the gland cells. In some of the larger gland cells there are apparently some double nuclei present. The connective tissue between the acini shows loose texture; it is rich in fibroblasts and contains some polynuclear leukocytes and lymphocytes and plasma cells.

We find then at this stage a secreting gland in which, however, the stimulus present during pregnancy is still active and causes slight proliferation.

Guinea Pig 2.—15 days after delivery; new pregnancy; the animal suckles her young. Much milk is present in the gland; the acini have a rather large lumen and in some cases there is a fine, loosely arranged precipitate, and in these acini the gland cells are generally flat and vacuolar. In other acini the vacuolar cells are a little higher. The nuclei form sometimes only a narrow band around the vacuoles. Also whole gland cells, with their nuclei, apparently disintegrate in the process of milk production. Some acini show irregular projections into the lumen. The acini are separated from each other by these strands of dense fibrous tissue with small nuclei. Mitoses are absent in the gland cells.

Guinea Pig 3.—19 days after delivery; new pregnancy; the animal suckles her young. This gland is similar to that in Guinea Pig 2, but the epithelial cells are, on the average, a little higher, especially in one acinus where no secretion is present. In some of the acini where secretion is present, some of the cells are pressed flat. Some cells apparently have two nuclei. In other cells vacuolization takes place and many cells swell and degenerate. In this case there is less connective tissue present than in Guinea Pig 2, and the connective tissue is richer in cells, which are larger and have larger vesicular nuclei. Mitoses are absent in this gland. A number of gland cells are cast off; occasionally a cast off cell has two nuclei. We find occasionally cell inclusions consisting of degenerated cells.

Guinea Pig 4.—29 days after delivery; new pregnancy; the animal suckles her young. This specimen represents a gland in the last period of secretion. The acini have wide lumina. Many vacuoles are visible in the gland cells. The nuclei are, on the whole, pale, and nuclear debris is seen in many acini; evidently

many cells degenerate. No mitoses can be found, but apparently a few double nuclei occur in gland cells. There is little connective tissue between the acini, but a few lymphocytes and polynuclear leukocytes are present.

We see then that after the completion of pregnancy and in the beginning of secretion, some mitotic proliferation may still be present, but that it soon ceases, probably as the result of those processes that lead to secretion. Thus while during the period of secretion, in spite of the presence of pregnancy, mitotic proliferation ceases, and the process of secretion is able to counteract the stimulating effect of pregnancy, nevertheless some proliferative stimulus appears to be active, which, however, under the conditions present during secretion, apparently merely leads to a mitotic multiplication of nuclei. This conclusion is suggested by the appearance of double nuclei in a number of gland cells. We intend, however, to investigate further the occurrence of double nuclei in secreting glands before we consider a mitotic nuclear division in secreting cells of the mammary gland as established. It also appears that during the process of secretion not only parts of the cells are transformed into material to be secreted, but that whole cells degenerate and are admixed to the secreted material.

Not only during proliferation, but also during secretion the formation of a large quantity of dense fibrous tissue is prevented in the mammary gland.

Guinea Pig 5.—25 days after delivery; new pregnancy; the animal does not suckle her young. The gland is large, but the acini are small, and they have only small lumina. The epithelium is cuboidal. In some of the larger acini some colloid-like material is present. No mitoses are seen. The connective tissue between the acini is highly cellular; dense fibrous tissue has not been produced. Some polynuclear leukocytes are present in the interstitial tissue. This gland is, therefore, still in an intermediate condition, but might have been expected within a short time to begin to proliferate.

Mammary Gland after Abortion.

In eight animals the mammary gland was examined soon after abortion had begun or had been completed. In all these cases some experimental procedure had taken place previously, which probably led directly or indirectly to the abortion.

In five instances the abortion took place between the 17th and 22nd days of pregnancy. In these cases the mammary gland did not present the picture of a secreting gland, but was either resting, intermediate, or proliferating. In two cases abortion took place in the latter part of pregnancy, 49 to 51 days after copulation. In guinea pigs the normal duration of pregnancy is approximately 65 to 66 days. In these cases the abortion produced secretion in the mammary gland, and microscopically the typical picture of secretion was present. We may therefore conclude that after proliferation has proceeded for a certain time, inhibition of the proliferative stimulus of pregnancy leads to such changes, of a more or less degenerative character in the gland cells, as precede and cause secretion. One of the animals (No. 6) again demonstrates the fact observed after the normal completion of pregnancy that at an early period of secretion there may still be present some cell proliferation in the secreting mammary gland.

The eight cases observed are briefly as follows:

Guinea Pig 7.—After incomplete extirpation of the corpora lutea, abortion took place 18 days after copulation; resting gland.

Guinea Pig 8.—Cuts had been made into the ovaries; abortion took place 19 days after last ovulation; resting gland.

Guinea Pig 9.—After incomplete extirpation of the corpora lutea, abortion had taken place 17½ days after previous ovulation; partly proliferating gland.

Guinea Pig 10.—After thyroidectomy and incomplete extirpation of the corpora lutea, abortion took place. Examination 22 days after last ovulation; intermediate gland.

Guinea Pig 11.—In a similar instance in which the mammary gland was partly proliferating, abortion had taken place only in one horn, while the other was pregnant. This case will be referred to below.

Guinea Pig 12.—Two intravenous injections of hirudin had been given to a pregnant guinea pig; beginning abortion; a proliferating gland was found. In this case the stage of pregnancy at the time of abortion is not certain.

Guinea Pig 6.—On the 49th day of pregnancy cuts were made into a mammary gland. Abortion took place. The gland was proliferating as well as secreting. Frequent mitoses were found in those areas in which there was no secretion in the acini; where there was some secretion, mitoses were still present; mitoses were seen even in acini much distended by secretion. In different lobules the degree of secretion and proliferation varied. The connective tissue was rather cellular; some polynuclear leukocytes and lymphocytes were present in the stroma. It is possible that the incisions in the uterus, made previous to abortion, were

in this case responsible for the mitotic proliferation; perhaps the stimulus supplied by pregnancy caused it.

Guinea Pig 13.—A large secreting gland without mitoses; abortion was beginning on the 41st day of pregnancy.

Effect on the Mammary Gland of Extirpation of the Ovaries during Pregnancy.

The mammary gland was examined in four guinea pigs in which the ovaries had been extirpated $3\frac{1}{2}$ to $6\frac{1}{2}$ days after copulation, and in which notwithstanding the absence of the ovaries, the pregnancy continued for some time. The mammary gland was examined $17\frac{1}{2}$ to $18\frac{1}{2}$ days after copulation. In three cases there was beginning abortion; in one of these abortion existed as yet only in one horn of the uterus, while in the other horn pregnancy still proceeded. In the fourth animal, in which the ovaries had been extirpated $5\frac{1}{2}$ days after copulation, a normal pregnancy was still present 13 days after castration. In these four instances the gland was either resting or intermediate. Proliferation was absent. From these data we can draw no definite conclusions as to the direct effect of placenta or embryo on the mammary gland, because during the stage of pregnancy at which examination took place, proliferation in the mammary gland is often absent even in normal animals.

Mammary Gland in Pregnant Animals after Extirpation of the Corpora Lutea.

In seventeen animals the corpora lutea were extirpated in most cases about 6 days, in some cases 3 to 4 days after copulation. Pregnancy proceeded usually until the time of examination, which took place 14 to $18\frac{1}{2}$ days after copulation; in some cases abortion had taken place previous to, or was proceeding at the time of the examination. We can arrange these seventeen cases into two classes according to whether or not a new ovulation had taken place at the time of examination. In the first class (*a*) comprising seven animals no new ovulation had taken place; in the second class (*b*) comprising ten animals, a new ovulation had taken place at the time of examination.

Animals in Which a New Ovulation Had Not Taken Place at the Time of Examination.—In three of these cases pregnancy was still present from $15\frac{1}{2}$ to $18\frac{1}{2}$ days after copulation, and after an absence of the corpora lutea during a period of 9 and 12 days. In three other cases abortion was just taking place, and in the last animal abortion had been completed at the time of examination. In three animals the mammary gland was intermediate, in three others not quite intermediate, and in one the mammary gland was somewhat better than intermediate, but not proliferating. In this case almost mature follicles were present in the ovaries, and it is possible that a new period of heat was impending.

Animals in Which a New Ovulation Had Taken Place at the Time of Examination.—In three guinea pigs new ovulation had taken place within 2 days preceding examination, a period of the sexual cycle during which proliferation is usually present in the mammary gland. In the first one of these animals ovulation had taken place sometime between 6 and 18 hours previously; abortion was just proceeding; the mammary gland was in part proliferating. In the second abortion had been completed at the time of examination. A new ovulation had occurred about $1\frac{1}{2}$ days preceding examination. In this guinea pig it is not certain that mitoses were present in the mammary gland, but the structure of the gland indicated that some proliferation might have been present a short time previously. In the third case pregnancy was still present, ovulation had taken place about 2 days before examination; the mammary gland was proliferating. In these three animals the corpora lutea had been absent during a period varying between 8 and 11 days. These observations confirm conclusions formulated in the preceding paper. The period directly following ovulation is accompanied by proliferative changes in the mammary gland, even if the ovulation has been accelerated experimentally, with or without a concomitant pregnancy. These observations furthermore prove that abortion does not necessarily prevent these proliferative changes.

In seven other guinea pigs ovulation had taken place about 3 to 8 days preceding examination. All were still pregnant. In one case, examined 19 days and a few hours after copulation, incisions had been made into one horn of the uterus at the time of the extirpation

of the corpora lutea. Small deciduomata developed, of which one was still alive and one necrotic at the time of examination. A new ovulation had taken place about 7 days previously and corpora lutea had been absent during a period of 6 days. In this animal the mammary gland was slightly proliferating. In the other six the mammary gland was either resting or resting to intermediate. In these instances the pregnancy was 18 to 19 days old, and the corpora lutea had been absent during a period varying between 6 and 9 days. In four of these animals a new ovulation had taken place 3 to 6 days previous to examination; the mammary gland was resting to intermediate. In two cases a new ovulation had taken place 6 to 8 days previously; in these two animals the mammary gland was in a resting stage.

We see, therefore, that the same factors determine conditions in the mammary gland if cyclic changes have been induced experimentally during pregnancy and in cases in which the normal cyclic changes have taken place without pregnancy.

If we consider the cases in which, during pregnancy, the corpora lutea or ovaries had been extirpated and examination was carried out 15 to 20 days after the last ovulation, we find that in none of the animals examined was the gland proliferating, while during the corresponding stages of either the ordinary cycle without pregnancy or during the corresponding period of pregnancy, a certain proportion of glands was found proliferating. This makes it probable that in this case also extirpation of the corpora lutea or ovaries directly or indirectly inhibited proliferative changes in the mammary gland. Extirpation of the corpora lutea during pregnancy, however, does not prevent, but on the contrary accelerates the proliferative changes in the mammary gland concomitant with a new ovulation.

Effect of Injections of Lutein on the Mammary Gland.

It seemed important to test the effect of the corpus luteum on the mammary gland in still another way; namely, directly through injections of the corpus luteum substance into the peritoneal cavity of the guinea pig.[4] 0.5 to 1 gm. of lutein was suspended in a sterile

[4] For this purpose we used Armour and Company's preparation of lutein from the cow.

bottle in 10 to 12 cc. of distilled, sterilized water and shaken in a mixing apparatus during a period varying between $2\frac{1}{2}$ and 7 hours. The fluid was then centrifuged for a few minutes and thus the undissolved residue removed. The supernatant fluid was injected intraperitoneally; about 8 to 12 cc. of fluid were given each time to one animal. Twenty-two guinea pigs were thus injected and an interval of 2 or 3 days was allowed to elapse between two succeeding injections. In seven of the animals the ovaries had been extirpated, in eight the corpora lutea had been removed, in one of the latter incompletely. In four animals incisions had been made in the uterus; similar incisions had been made in the uterus in animals in which ovaries or corpora lutea had been extirpated. In three cases the injections were made during the second half of the sexual cycle without any other experimental interference with the animal. In a few instances in which incisions had been made in the uterus previous to the injections, smaller or greater parts of the uterus became infected as the result of the injections. Some bacteria were apparently present in the lutein specimens. After injections into an uninjured peritoneal cavity, they did not lead to infection, but sometimes they infected the surface of a cut uterus. Lutein injections as such did not produce proliferation of the mammary gland, although sometimes as many as five injections were given to the same animal.

In seven cases lutein was injected in animals in which both ovaries had been extirpated; at the same time incisions were made into the uterus. Two of the animals received five, one three, two two injections, and two one injection. In a few animals the first injection of lutein was given 1 day before castration. The animals were examined 11 to 15 days after the preceding heat. In none was proliferation noticeable in the mammary gland, which was either resting or varying between the resting and intermediate state.

Injections of lutein were given to eight animals in which the corpora lutea had been extirpated. In one the extirpation had been incomplete. In one case five, in two cases four, in one case three injections of lutein were given, and in three cases one injection was given. In several guinea pigs the first injection was given 1 or 2 days before the operation. In the majority of the animals incisions had been made in the uterus at the time of extirpation of the cor-

pora lutea; in two of these an infection of the cut uterus occurred, evidently as the result of the lutein injections. In these eight animals the examination took place at the following periods.

(1) 17 days after the preceding heat; five injections had been given; the mammary gland was resting. (2) 7 days and 17 hours after the preceding heat; extirpation was incomplete; one injection had been given; the mammary gland was intermediate. (3) 13½ days after the previous heat; four injections had been given; the mammary gland was resting. (4) 16½ days after the preceding heat; four injections had been given; the mammary gland was approximately resting. (5) 6 days after the preceding heat; 3 days after the lutein injection, one injection being given; the gland was intermediate. (6) 5 days after the preceding heat and 2 days after the lutein injection, one injection being given; the mammary gland was intermediate. (7) 10½ days after the preceding heat; small deciduomata were slightly infected at some places; one injection had been given; the mammary gland was resting. (8) 14 days after the preceding heat; three injections were given; the mammary gland was almost resting.

We see that the injections of lutein did not exert any noticeable influence on the mammary gland in animals in which the corpora lutea had been extirpated.

In four animals incisions in the uterus were made some time previous to the injections of lutein.

(1) Incisions were made in the uterus during the positive period (3 to 7 days after ovulation). 17½ days after heat the first injection of lutein was made; 26½ days after heat examination took place. Good deciduomata had developed, the corpora lutea were preserved. Four injections of lutein were given. The mammary gland was resting. (2) 6½ days after ovulation incisions were made into the uterus. 18½ days after ovulation one injection of lutein was given. Examination of the animal took place 21½ days after ovulation. The uterus, in which deciduomata had developed, was markedly infected; no mature follicles, but good corpora lutea were present in the ovaries; the mammary gland was proliferating. (3) Five injections of lutein were given; 25½ days after ovulation examination was made. Large deciduomata and corpora lutea which were on the whole preserved, but showed some peripheral vacuolization, were present. A new ovulation had not taken place. The mammary gland was intermediate. (4) Four injections of lutein were given; examination 26½ days after ovulation. 11 days after ovulation the first injection of lutein was given. Living deciduomata and small corpora lutea were found. The mammary gland was proliferating.

We find proliferating mammary glands in several cases of this series. But inasmuch as this might be expected even without lutein injections, we cannot ascribe this result to the injection of lutein.

In three cases injections of lutein were made in animals in which no experimental interference had taken place. The injections were made in the latter half of the sexual cycle.

(1) Five injections were given, the first one 13½ and the last 22½ days after heat; examination took place 23½ days after heat. No new ovulation had occurred. The mammary gland was resting. (2) Four injections were given, the first 15 and the last 22 days after heat; examination was made 24 days after heat. A new ovulation had occurred about 4 days before examination. The mammary gland was found to be intermediate. (3) Four injections were given, the first 12½ and the last 19½ days after heat; 23½ days after ovulation examination was made. A new ovulation had taken place 5 to 6 days previous to examination. The mammary gland was intermediate.

In one control case four injections of 14 cc. of 0.4 per cent sodium chloride solution were made intraperitoneally, the first injection being given 11½ days and the last 21½ days after ovulation; examination 22 days after the first heat showed that a new ovulation had occurred 2½ to 3 days previous to examination. The mammary gland was therefore found to be partly proliferating.

In these cases again results were obtained which had to be solely attributed to the condition of the sexual cycle. The mammary gland was apparently not influenced by the injection of lutein. We may then conclude that as many as five consecutive injections of lutein given to the same guinea pig do not exert a noticeable stimulating effect on the mammary gland. While it is possible that a still larger number might have produced a definite effect, we should have expected to notice microscopically at least a beginning proliferation after as many as five injections. But in no case could such an effect be established. Under normal conditions the action of the corpus luteum is probably continuous; under the conditions of our experiment the action was not continuous, although a relatively large quantity had been injected each time.

It is, furthermore, possible that the effective constituent of the corpus luteum possesses a certain species specificity, and that while the corpus luteum extract of the cow is ineffective, the extract of the guinea pig corpus luteum might have been effective. Of course we have to consider the fact that even during the normal cycle proliferation in the mammary gland takes place in the absence of the

corpus luteum and that on the other hand proliferation is absent at certain periods when corpora lutea are well developed. In this respect, then, the negative result of the injections of lutein agrees with what we found at certain periods in the normal animal. However, we found indications that the corpus luteum may favor proliferation of the mammary gland under certain conditions, and our experiments prove that as many as five injections of cow's lutein are unable to produce even the beginning of proliferation.

SUMMARY.

1. In the pregnant guinea pig proliferation of the mammary gland becomes regular only at a later stage of pregnancy; namely, during the period following the 24th day of pregnancy. Previous to this period proliferation was absent in the majority of cases. Proliferation of the mammary gland during pregnancy becomes regular only at a period of time which exceeds the duration of the normal sexual cycle unaccompanied by pregnancy. It is probable that pregnancy as well as the presence of living deciduomata and corpora lutea increases the proliferative activity of the mammary gland as compared with the ordinary cycle in non-pregnant animals or in animals lacking corpora lutea and deciduomata.

2. After the completion of pregnancy and in the beginning of secretion some mitotic proliferation may still be present, but it soon ceases, probably as the result of those processes that lead to secretion. While during the period of secretion, notwithstanding the presence of a new pregnancy, mitotic proliferation soon ceases, some proliferative stimulus seems still to be active, which, however, under existing conditions apparently leads only to a mitotic multiplication of nuclei. The latter conclusion is only suggested at the present time and needs confirmation through further studies.

3. In cases in which abortion took place in the first half of pregnancy secretion in the gland was not established; secretion occurred in two animals aborting toward the latter part of pregnancy. In one of these cases, mitotic proliferation of some gland cells was associated with the microscopic appearances of secretion.

4. In guinea pigs castrated during an early period of pregnancy

in which pregnancy continued for some time, proliferative changes were absent in the mammary gland. In conjunction with a partial similar effect observed after extirpation of the corpora lutea during pregnancy, we may perhaps attribute the lack of proliferation in some of these cases to the absence of the ovaries.

5. Extirpation of the corpora lutea during pregnancy induces a new ovulation and with it the primary proliferation in the mammary gland; abortion does not necessarily prevent these proliferative changes. Extirpation of the corpora lutea during pregnancy perhaps prevents the secondary proliferative changes in the mammary gland.

6. Five injections of cow's lutein given in relatively large quantities intraperitoneally do not produce proliferation of the mammary gland in the guinea pig.

Correlation of the Cyclic Changes in the Uterus and Ovaries with Those in the Mammary Gland.

We can distinguish two phases in the cycle of the ovaries and uterus; the first phase comprises the period of heat, ovulation, and the period immediately following ovulation. In this period certain growth processes take place in the uterus. The processes taking place in the uterus and ovaries during this period are dependent on the removal of an inhibiting substance produced by the corpus luteum and on the presence of a substance produced through another constituent of the ovaries, in all probability the large follicles including the mature follicles or perhaps the latter alone. As we pointed out previously, an interstitial gland comparable to that of the rabbit does not exist in the guinea pig. Heat changes therefore depend on the absence of the corpus luteum and on the presence of another ovarian constituent.

This period is followed by the second and longer period dominated by the function of the corpus luteum. As we have shown experimentally, the secretion of the corpus luteum sensitizes the uterine mucosa in such a way that the decidual and predecidual proliferation takes place, the former after application of mechanical stimuli or after the injury produced through the embryo penetrating into the mucosa, the latter under the influences of ordinary metabolic

changes. This sensitizing influence of the corpus luteum is, however, not exerted throughout the whole remaining part of the sexual cycle, but only during a period of 6 or 7 days (from the 3rd to the 9th or 10th day after ovulation). We must either assume that the corpus luteum ceases to secrete this sensitizing substance after the 9th day after ovulation or else that after this period it secretes in addition a substance inhibiting the action of the first substance. We have shown that the corpus luteum has an inhibiting action by force of which it prevents the appearance of a new ovulation and heat. This latter substance is given off during the whole period of the sexual cycle. It is therefore probable that we have to deal with two different substances secreted by the corpus luteum.

During pregnancy conditions are essentially the same as without pregnancy. The corpus luteum exerts the same two functions. There is only one essential difference between the conditions during pregnancy and without pregnancy. If we accelerate experimentally the onset of a new sexual cycle without pregnancy, the newly formed corpus luteum causes also a new cycle in the uterine mucosa, while during pregnancy the experimental production of a new corpus luteum is not followed by a new cycle in the uterine mucosa; here some inhibiting mechanism is at work. If we now correlate the cyclic changes in the mammary gland with those in the uterus and ovaries, we find proliferative changes in the mammary gland corresponding to the first phase of the ovarian and uterine cycle. We may call this the primary growth period of the mammary gland. We have every reason to believe that the swelling of the human mammary gland during menstruation, observed by many and in the absence of microscopic studies referred to vascular changes, represents in part these growth processes.

We are able to accelerate experimentally the primary proliferation of the mammary gland; in the same way we can experimentally accelerate heat and ovulation, through removal of the inhibiting tonus exerted chemically by the corpus luteum. The primary growth period of the mammary gland can be experimentally induced as well during pregnancy as without pregnancy. Contrary to what might have been expected, we find that the growth stimulus exerted on the uterus by the corpus luteum in the first part of the second phase of

the sexual cycle does not find a counterpart in the mammary gland of the guinea pig. During the whole period of the development and activity of the corpus luteum of the non-pregnant animal the mammary gland in the majority of cases is inactive. Only during the last part of the sexual cycle may we find in some cases some proliferation of the mammary gland. Whether this is due to the approach of the succeeding period of heat or is an accumulative late effect of the corpus luteum substance cannot be determined with certainty at present. It is possible that a quantitatively stronger action of the corpus luteum at last produces proliferative changes also in the guinea pig. Thus we usually found proliferation in cases in which we artificially prolonged the period of the sexual cycle and in which well preserved corpora lutea and deciduomata were present. How much in these cases the proliferation of the mammary gland has to be attributed to a quantitatively stronger action of the corpus luteum or to the deciduoma or to the combination of both, cannot be decided definitely at the present time.

The conditions are similar during pregnancy. We find here definite growth of the mammary gland only after the passing of a period which is longer than the duration of the normal sexual period. But in a number of instances we find following the 12th day of pregnancy some proliferation in the mammary gland. Pregnancy as well as the presence of living deciduomata and well preserved corpora lutea causes an increase in the number of proliferating glands during a time corresponding to the closing period of the normal sexual cycle. We might designate this as the secondary growth period of the mammary gland. In the pregnant animal it develops into the typical hypertrophy of the mammary gland characteristic of this condition, and, indeed, represents merely its initial stage.

In the rabbit Ancel and Bouin[5] as well as Frank and Unger[6] noticed a parallelism between the presence of good corpora lutea and the

[5] Ancel, P., and Bouin, P., *J. physiol. et path. gén.*, 1911, xiii, 31.

[6] Frank, R. T., and Unger, A., *Arch. Int. Med.*, 1911, vii, 812. Shortly after the preliminary communication of our results (*Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 91) there appeared a preliminary note by M. Athias (*Compt. rend. Soc. biol.*, 1916, lxxix, 557) in which this author described growth and secretion in the mammary gland in castrated male guinea pigs, into which ovaries had been transplanted. At the time of examination, soon after the beginning of lactation,

growth of the mammary gland. Ancel and Bouin conclude that it is the corpus luteum which determines the growth of the mammary gland. Our investigations show that in the guinea pig the factors determining the growth of the mammary gland are complex, and that they can be considered only partly analyzed at the present time. Provided the facts observed by Ancel and Bouin in the rabbit have found a correct interpretation, we must assume that in the rabbit the response of the mammary gland to the secretions of the corpus luteum is much more prompt than in the guinea pig. We would have to assume that the gland cells in the rabbit are more labile and thus react at once, while in the guinea pig the stimulus has to accumulate during a considerable period of time before a reaction sets in. From a biological point of view such a difference between the rabbit and guinea pig could well be understood if we consider the fact that in the rabbit the duration of pregnancy is much shorter than in the guinea pig, and that therefore the service of the mammary gland is required in the rabbit at a much earlier period after copulation than in the guinea pig. While such a consideration is not a causal explanation, it renders it at least plausible that a marked difference in the power of reaction of the two kinds of mammary gland does exist.

As to the secretory function of the mammary gland, we are inclined to consider it as a phenomenon intermediate between growth on the one hand and complete degeneration on the other hand, as we find it with deciduomata and under certain conditions also in the mammary gland[7] after the growth stimuli have suddenly ceased to act. It seems that the regenerating effect of all divisions is necessary in certain tissues in order to insure their continued preservation. The peculiar constitution of the mammary gland causes instead of complete degeneration, an intermediate state of intercellular degeneration, which is, however, coupled with a degeneration of a considerable number of gland cells. Such a view is somewhat akin to that of Hildebrandt[7] without, however, being identical with the latter.

there were no well developed corpora lutea in the ovaries. The author concludes that follicles or interstitial gland, but not the corpora lutea are responsible for the growth of the mammary gland. (It is not our intention to consider extensively the literature on this subject at the present time; we may give a more complete review at a later date.)

[7] Hildebrandt, P., *Beitr. chem. Phys. u. Path.*, 1904, v, 463.

A COMPARATIVE STUDY OF CERTAIN ACTIONS OF ADRENALIN IN THE CAT AND THE RABBIT.

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PLATES 29 TO 31.

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After unilateral excision of the superior cervical ganglion, certain substances or conditions produce an effect on the side on which the ganglion has been removed, while on the other side on which nothing has been done, or on which the sympathetic nerve has been cut below the ganglion, the pupil is affected but little or remains comparatively unaffected. This is called paradoxical pupil dilatation.

Meltzer and Auer[1] found that subcutaneous injection or instillation in the conjunctival sac, which has no effect upon the pupil of the normal rabbit, produces considerable dilatation of the pupil in the rabbit on the side on which the superior cervical ganglion has been removed, while the pupil on the side on which only the sympathetic nerve has been cut, remains unchanged; in other words, adrenalin causes a paradoxical pupil dilatation in the rabbit. Meltzer[2] found later that the same holds true for the cat, but only when adrenalin is administered by subcutaneous injection. He has not been successful with instillation of adrenalin into the eyes of operated cats, on account, he says, of the "protruding nictitating membrane which would sweep over the entire eye as soon as adrenalin came in contact with it, thus removing the adrenalin." Meltzer adds that he has not been persistent enough in his attempts.

In a series of experiments on cats we have made many attempts to produce dilatation by instillation of adrenalin in the conjunctival sac on the side on which the ganglion was previously removed and have found that it is difficult to produce dilatation in the cat by this method. While in rabbits dilatation of the pupil is readily obtained

[1] Meltzer, S. J., and Auer, C. M., *Am. J. Physiol.*, 1904, xi, 28.

[2] Meltzer, S. J., *Am. J. Physiol.*, 1904, xi, 37.

by a single instillation, in cats we obtained in many cases a moderate dilatation only after many instillations in the course of 2 hours, and in many instances we failed. In addition to the action of the nictitating membrane mentioned by Meltzer, it is possible that the conjunctiva of the cat is more dense and does not favor absorption.

However, it seemed doubtful that these mechanical factors alone could sufficiently account for the failure of conjunctival instillation, in view of the ease with which eserine and atropine cause dilatation of the pupil in the cat. There seemed to be no obvious reason why adrenalin should be absorbed less readily than these substances, and we thought that there might be other reasons for the difference in the response of the two species of animals.

Our first problem was to establish whether the responsiveness of the iris differed in the two species, and we believed that some light might be thrown on the matter by studying the effect of adrenalin, given by intravenous injection, on the cat's pupil and comparing this effect with that of similar injections in the rabbit. By this means all differences in the degree of conjunctival absorption would be eliminated.

The solution used for the injection was made by dissolving adrenalin crystals in 0.7 per cent sodium chloride with hydrochloric acid, and diluting until an accurate 0.1 per cent solution was obtained. For the smaller doses this was further diluted before injection. In this way we had a reliable solution at our disposal throughout the experiments.

All the animals used had undergone excision of the superior cervical ganglion, at least several days, and often several weeks before the experiments. Notes were made on the pupil of the intact, as well as on that of the operated side.

The rabbits were either held gently without narcosis and the injection was given into the marginal vein of the ear, or the jugular vein was exposed after anesthetizing the skin with ethyl chloride and the injection given into the vein. No difference was observed between the results of injections into the jugular and the ear vein. Cats were always injected in the jugular vein.

There is some difficulty in obtaining records with cats, on account of the paradoxical dilatation which appears so readily in excited

animals after gangliectomy. If the cat is stroked gently during the operation, it becomes quiet and the pupils return to normal. If the needle is then inserted in the vein and a few seconds are permitted to elapse before the injection is given, no dilatation results from the manipulation alone, as we have observed repeatedly by injection of saline solution. In a few instances the adrenalin caused marked disturbance of heart action with dyspnea. The results of these injections were discarded.

The present study deals exclusively with the size of the pupil; other effects of adrenalin on the eye were disregarded in the protocols.

The response of the pupil to intravenous injection of adrenalin has been studied in the cat by Lewandowsky[3] and in the rabbit by Joseph.[4] Lewandowsky made only qualitative observations and gives no figures either as to dosage of the adrenal extract or the degree of dilatation of the pupil which it produces. Joseph studied the quantitative response of the pupil of the rabbit after gangliectomy, but his observations were limited to the operated side. In the main, our results agree with Joseph's, the only difference being that in our experiments the response of the rabbit's pupil to a given dose was greater, both in degree and duration.

Our results may be briefly stated as follows: In the rabbit the dilating influence of intravenous injections of adrenalin was not seen in the eye on the unoperated side with doses smaller than 0.05 mg. per kilo of body weight; with doses less than 0.1 mg. per kilo the effect was not constant and when manifest was slight and lasted only a few minutes. A dose of 0.1 mg. per kilo of body weight caused, as a rule, moderate or slight dilatation with return to normal in about 10 minutes. In two animals no dilatation was seen on the intact side from this quantity. Dilatation of the pupil of the gangliectomized side was noted with doses of 0.01 mg. per kilo of body weight. With doses below 0.02 mg. however, the effect was slight and inconstant. The most constant results were obtained with a dose of 0.1 mg. With this dose a maximal dilatation ensued which lasted about 10 minutes, followed by a gradual return to normal in the course of 2 or 3 hours.

[3] Lewandowsky, M., *Arch. f. Physiol.*, 1899, 360.

[4] Joseph, D. R., *J. Exp. Med.*, 1912, xv, 644.

In the cat the response was found to be much greater. On the eye of the intact side doses of adrenalin as small as 0.01 mg. per kilo of body weight gave a distinct and constant effect, and a dose of 0.1 mg. gave rise to almost maximal dilatation. The duration was, however, brief even with the largest dose, the pupil becoming normal within 5 minutes. Similar relations were found on the operated side. Here minute doses, even as small as 0.002 mg. per kilo of body weight, caused a distinct dilatation, and 0.01 mg. per kilo gave rise to almost maximal dilatation. In both cases the dilatation was transient. A dose of 0.1 mg. per kilo of body weight caused maximal dilatation lasting about 5 minutes with a gradual return to normal within half an hour.

ILLUSTRATIVE PROTOCOLS.

Rabbit 1.—White female; weight 1.480 kilos.

Jan. 21, 1916. Right superior cervical ganglion excised under ether anesthesia.

Mar. 29, 9.21 a.m. Injected in ear vein, 1 cc. of adrenalin, 1 : 50,000, (0.0135 mg. per kilo).

Before injection,				right	pupil	5	mm.	in	diameter;	left	5	mm.
½	min.	after	injection,	"	"	8	"	"	"	"	5	"
1½	"	"	"	"	"	8	"	"	"	"	5	"
2	"	"	"	"	"	7	"	"	"	"	4½	"
4	"	"	"	"	"	5½	"	"	"	"	4½	"
5	"	"	"	"	"	5	"	"	"	"	5	"

Apr. 6, 10.00 a.m. Injected intravenously 0.15 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).

Before injection,				right	pupil	5	mm.	in	diameter;	left	5	mm.
½	min.	after	injection,	"	"	9	"	"	"	"	6	"
3	"	"	"	"	"	9	"	"	"	"	6	"
5	"	"	"	"	"	8	"	"	"	"	3	"
10	"	"	"	"	"	6½	"	"	"	"	4	"
15	"	"	"	"	"	7	"	"	"	"	5	"

Rabbit 2.—White female; weight 1.910 kilos.

Jan. 21, 1916. Right superior cervical ganglion excised under ether anesthesia.

Apr. 5, 3.23 p.m. Injected intravenously 0.19 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).

Before injection,				right	pupil	4	mm.	in	diameter;	left	4	mm.
½	min.	after	injection,	"	"	8½	"	"	"	"	3½	"
1	"	"	"	"	"	8	"	"	"	"	3½	"
3	"	"	"	"	"	8	"	"	"	"	5	"

10	min. after injection,					right pupil 10	mm. in diameter;			left 3	mm.
17	"	"	"	"	"	10	"	"	"	" 4	"
57	"	"	"	"	"	8½	"	"	"	" 3	"
77	"	"	"	"	"	6½	"	"	"	" 3	"
100	"	"	"	"	"	5½	"	"	"	" 4	"
112	"	"	"	"	"	4	"	"	"	" 2	"

Cat 1.—Black male; weight 2.660 kilos.

Mar. 27, 1916. Left superior cervical ganglion excised under ether anesthesia.

Mar. 30, 10.13 a.m. Injected intravenously 2 cc. of adrenalin, 1 : 50,000 (0.014 mg. per kilo).

Before injection,					left pupil a slit;					right a slit.	
½	min. after injection,				" "	10	mm. in diameter;			" 4	mm.
1	"	"	"	"	"	6	"	"	"	" 2	"
2	"	"	"	"	"	1	"	"	"	" 1	"

10.23 a.m. Second injection. 2 cc. of adrenalin intravenously, 1 : 50,000 (0.014 mg. per kilo).

Before injection,					left pupil	1	mm. in diameter;			right	1 mm.
½	min. after injection,				" "	10	"	"	"	" 2	"
1	"	"	"	"	"	8	"	"	"	" 1	"
2	"	"	"	"	"	2	"	"	"	" slit.	
7	"	"	"	"	"	1	"	"	"	" 1 mm.	

10.45 a.m. Third injection. 2 cc. of adrenalin intravenously, 1 : 10,000 (0.07 mg. per kilo).

Before injection,					left pupil	1	mm. in diameter;			right	1 mm.
½	min. after injection,				" "	15	"	"	"	" 10	"
1	"	"	"	"	"	15	"	"	"	" 8	"
2	"	"	"	"	"	12	"	"	"	" 6	"
3	"	"	"	"	"	12	"	"	"	" 2	"
4	"	"	"	"	"	10	"	"	"	" 1	"
6	"	"	"	"	"	6	"	"	"	" 2	"

Apr. 10. Weight unchanged.

10.09 a.m. Injected intravenously 0.285 cc. of adrenalin, 1 : 1,000 (0.11 mg. per kilo).

Before injection,					left pupil	3	mm. in diameter;			right	4 mm.
½	min. after injection,				" "	16	"	"	"	" 14	"
1	"	"	"	"	"	16	"	"	"	" 10	"
1½	"	"	"	"	"	16	"	"	"	" 6	"
2	"	"	"	"	"	14	"	"	"	" 3	"
6	"	"	"	"	"	10	"	"	"	" 3	"
8	"	"	"	"	"	6	"	"	"	" 3	"
18	"	"	"	"	"	2	"	"	"	" 2	"

Cat 2.—Gray male; weight 3 kilos.

Mar. 27, 1916. Left superior cervical ganglion excised under ether anesthesia.

Apr. 10, 10.30 a.m. Injected intravenously 3 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).

Before injection,				left pupil	3	mm.	in	diameter;	right	4	mm.
½	min.	after	injection,	"	17	"	"	"	"	12	"
1	"	"	"	"	17	"	"	"	"	4½	"
3	"	"	"	"	17	"	"	"	"	2½	"
5	"	"	"	"	17	"	"	"	"	2½	"
7	"	"	"	"	16	"	"	"	"	2	"
9	"	"	"	"	7	"	"	"	"	2	"
15	"	"	"	"	4	"	"	"	"	3	"
20	"	"	"	"	4	"	"	"	"	4	"
30	"	"	"	"	2	"	"	"	"	3	"

These examples illustrate the type of dilatation seen in the cat and in the rabbit after intravenous injections of adrenalin. The striking differences are the greater degree and briefer duration of the dilatation in the cat. This is true both for the normal and gangliectomized sides, and with all doses.

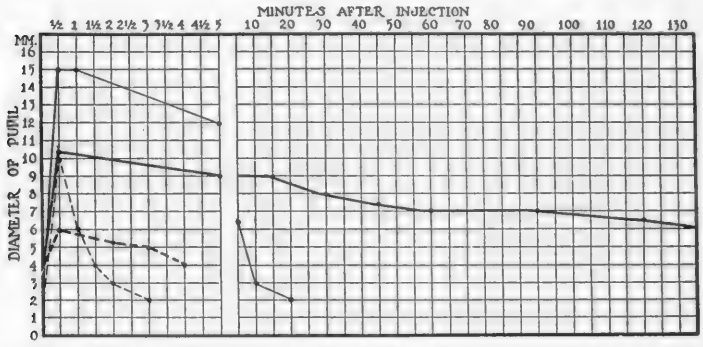
The following table shows the number of experiments and the average results.

Species.	Dose per kilo.	No. of experiments.	Pupil.	Time before return to normal.	Increase.	Maximum size of pupil.
	mg.			*min.*	*mm.*	*mm.*
Rabbit.....	0.01	3	Intact side.		0	4
			Operated side.	10	2	6
Rabbit.....	0.1	12	Intact side.	3	2	6
			Operated side.	180	6	10
Cat.....	0.01	2	Intact side.	1½	7	9
			Operated side.	10	13	15
Cat.....	0.1	13	Intact side.	2	8	10
			Operated side.	30	13	15

Text-fig. 1 shows graphically the course of the dilatation in the normal and gangliectomized pupils of rabbits and of cats, after injection of 0.1 mg. of adrenalin per kilo.

The cat's pupil, when fully dilated, is from 14 to 16 mm. in diameter, and the rabbit's from 10 to 12 mm. It will be seen that in the cat the pupil on the operated side showed approximately complete dilatation from even the smaller dose, while the rabbit showed full dilatation with the larger dose, but not even approximating it with

the smaller. The pupil of the normal side of the cat also showed a greater response to adrenalin than that of the rabbit. Comparison of the increase in diameter of the pupil of the intact side with that of the operated side, in each species, might seem to show a greater increase in response following gangliectomy in the rabbit, but this is deceptive and due to the marked effect on the pupil of the unoperated side of the cat, which would render a dilatation three times as great, such as occurs in the rabbit, an impossibility.



TEXT-FIG. 1. The course of dilatation of the pupil after intravenous injection of 0.1 mg. of adrenalin per kilo. Broken lines indicate the intact side; unbroken, after gangliectomy. Light lines show the course in cats; heavy lines, in rabbits.

Our original problem, namely, the question whether the difference in response of the two species to instillation of adrenalin was dependent on a greater responsiveness of the iris of the rabbit, seems, therefore, to be satisfactorily answered. Our experiments seem to show definitely that this is not the case. With intravenous injections it was found that the opposite was true; that is, that the responsiveness of the iris of the cat was greater than that of the rabbit. In other words, a given dose of adrenalin, injected intravenously, will cause wider dilatation of the pupil in the cat than in the rabbit.

The experiments brought out, however, the fact that while the degree of dilatation of the pupil which is brought about by a given dose of adrenalin is greater in the cat than in the rabbit, the duration

of the dilatation is, on the contrary, longer in the rabbit than in the cat. The question arose whether these inverse relations are confined to the iris, or whether it would be met also in the various other effects of adrenalin on the functions of the body.

The most readily observed and best known action of adrenalin is its constricting power on the arterioles. We therefore made a study of the influence of intravenous injections of adrenalin on the blood pressure in the cat and the rabbit, comparing the intensity of the reaction with the duration of the rise in the two species of animals.

The technique in this series of experiments differed from that already described in that all the animals were kept under light narcosis throughout the experiments.

The results showed that with all doses from 0.01 to 0.3 mg. the average effect upon the blood pressure was greater in the rabbits than in the cats, both in regard to the height and the duration of the rise. The actual height reached was greater in the cats, as their original blood pressure was higher, varying from 100 to 110 mm., while in the rabbits the normal blood pressure varied from 60 to 70 mm. With 0.1 mg. of adrenalin per kilo of body weight there was in rabbits an average rise of 85 mm. (actual maximum 150 mm.) with a return to normal in about 6 minutes (Fig. 1); in cats the rise was about 75 mm. (actual maximum 175 mm.) lasting about $3\frac{1}{2}$ minutes (Fig. 2). With 0.03 mg. per kilo, rabbits showed a rise of 70 mm. (maximum 135 mm.) lasting 4 minutes (Fig. 3); cats showed a rise of 45 mm. (maximum 150 mm.) lasting $3\frac{1}{2}$ minutes (Fig. 4). With 0.01 mg. rabbits showed a rise of 55 mm. (maximum 120 mm.) lasting 3 minutes; cats showed a rise of 35 mm. (maximum 140 mm.) lasting 2 minutes.[5]

It will be seen that the influence of adrenalin on blood pressure differs from that on the iris. In the iris the duration of the effect was at variance with the degree of dilatation, the one being greater in the rabbit the other in the cat. With blood pressure we find that the two differ in the same direction, both the duration and intensity of the effect being greater in the rabbit.

[5] In the rabbits the return to the base-line signalized the end of the response. In more than half the cats the return to the base-line was followed by a fall of from 10 to 40 mm. below the line with a gradual return to normal in from 2 to 10 minutes.

DISCUSSION.

The original problem was to determine whether there were other explanations than that suggested by Meltzer to account for the difference of response of the iris of the cat and rabbit to instillations of adrenalin. One possibility was that the iris of the rabbit was more sensitive to this substance. The experiments with intravenous injections showed, however, that the opposite was the case; namely, that the iris of the cat is more sensitive to adrenalin than that of the rabbit.

In these experiments it was, however, found that with regard to the pupil there was a contrast between responsiveness and duration. While in the cat the responsiveness was greater than in the rabbit, the duration of the effect was greater in the rabbit than in the cat. This led to the investigation of the effect of adrenalin on blood pressure in the two species to determine whether it showed the same relation. Here it was found on the contrary, that the responsiveness of the vasoconstrictors as well as the duration of the response was greater in the rabbit than in the cat.

It is possible that the brevity of the dilating effect upon the cat's pupil, seen in the intravenous injections of adrenalin, is an additional factor in the very slight effect of adrenalin on the pupil of these animals, when administered by instillation. The slight absorption which perhaps actually takes place produces no perceptible effect on account of the lack of a cumulative action.

SUMMARY.

The present investigation brought out the facts that the pupil of the cat is little affected by instillation of adrenalin, but shows, nevertheless, a greater responsiveness to adrenalin when given intravenously than does that of the rabbit, and that the duration of the dilatation effected by intravenous injection is, on the contrary, longer in the rabbit.

In regard to the vasoconstricting effect of adrenalin when administered intravenously, it was found that the intensity as well as the duration of the rise of blood pressure was greater in the rabbit than in the cat.

EXPLANATION OF PLATES.

Blood pressure of cats and rabbits after intravenous injection of adrenalin.

The upper line shows the blood pressure; the lower line, the base-line with the time in seconds and minutes. The numbers above the base-line show the transverse diameter of the pupil on the gangliectomized side. In Fig. 1 the upper row of numbers shows the normal eye, the lower row, the operated side.

PLATE 29.

FIG. 1. Rabbit 4. Injection of 0.1 mg. per kilo of body weight.

PLATE 30.

FIG. 2. Cat 4. Injection of 0.1 mg. per kilo of body weight.

PLATE 31.

FIG. 3. Rabbit 3. Injection of 0.03 mg. per kilo of body weight.
FIG. 4. Cat 3. Injection of 0.03 mg. per kilo of body weight.

FIG. 1.

(Githens: Adrenalin in the Cat and the Rabbit.)

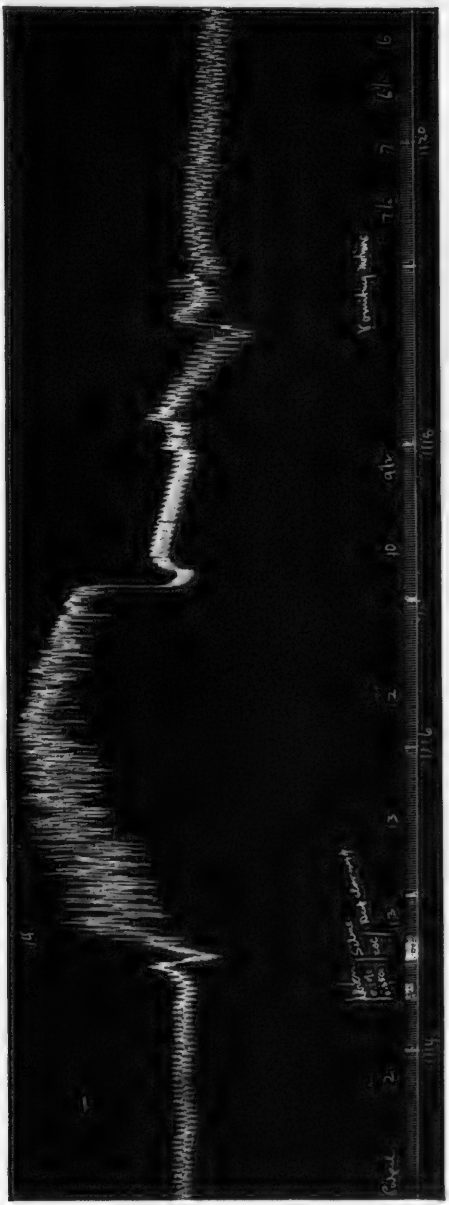


FIG. 2.

(Githens: Adrenalin in the Cat and the Rabbit.)

FIG. 3.

FIG. 4.

(Githens: Adrenalin in the Cat and the Rabbit.)

THE SIGNIFICANCE OF THE EPITHELIAL CELLS AND SAPROPHYTES IN SPUTUM.

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PLATES 32 AND 33.

(Received for publication, October 21, 1916.)

Although text-books on diagnosis state that the source of epithelial cells in the sputum may be ascertained by their microscopic appearance, the significance of this fact is not emphasized.

The mouth and pharynx are lined with polygonal flat epithelial cells, and the vocal cords are covered with them, while the nasal cavities, larynx, trachea, and bronchi are lined with ciliated cylindrical epithelial cells. The bronchioles and bronchi are lined with cuboidal cells, while the alveoli are lined with polygonal cells[1] which, unlike those of the other organs, have no nuclei. If inflammation occurs in any of these organs, the cells are expectorated with the mucus, and the sputum must therefore contain the epithelial cells of the affected organ. But in chronic inflammation of the larynx, as in tuberculosis, the ciliated epithelial cells lining this cavity are supplanted by a new growth of polygonal flat epithelial cells like those of the mouth. This is shown by the continual occurrence of polygonal flat cells in the sputum of laryngeal tuberculosis. The ciliated cells are distributed over a wide area, but they scarcely ever appear in the sputum. I have only once detected ciliated cells. In sputum from the lungs round or elliptical epithelial cells, which are nearly twice as large as pus cells, are often present. They are the decomposed products of the cuboidal cells found in the bronchioles. They have vesicular nuclei and contain either particles of carbon or hemosiderin in their protoplasm, and correspond to

[1] Some authors believe that these cells are enlarged cuboidal cells, and not epithelial cells.

Wagner's heart failure cells. I have never found flat pulmonary epithelial cells without nuclei in sputum.

The saprophytes play an important part in determining the source of sputum. In spite of the fact that various kinds of cocci, bacilli, spirochetes, and spirilla are found in the nasal and mouth cavities and in the larynx and pharynx, the sputum from the trachea and lungs is always free from these non-pathogenic microorganisms. The polygonal flat epithelial cells are found with various saprophytes, while the sputa that come from the lungs or trachea contain only the agent of the prevailing disease, such as *Bacillus tuberculosis* or *Diplococcus pneumoniæ*. In cases of mixed infection, one or two species of bacteria are found. Large numbers of various kinds of microorganisms in the pulmonary or tracheal sputum were never demonstrated.

I shall refer to the sputum from the region below the vocal cords as pulmonary sputum, that from above, as laryngeal sputum, and the exudates of the nasal cavity as nasal secretions.

Technique.

The same patient sometimes expectorates sputa of different appearance, revealing the various degrees of inflammation or other conditions of the affected parts. All the different sputa must therefore be examined, and the material for examination must be fresh and moist. A specimen attached to a piece of paper or gauze is unsuitable, as shown by the following experiment. A film preparation of a specimen of sputum to which some powdered starch had been added was pressed gently with a piece of blotting paper. The specimens were treated with iodine, stained with dilute fuchsin, and examined under the microscope. The particles of starch were found to be completely mixed with the sputum. On the other hand, the sputum that had been mixed with the starch suspension in salt solution and stirred vigorously proved on microscopical examination to have been mixed completely, and when subjected to the method of washing shown below, no particles of starch were demonstrated. This shows that the dehydrated starch grains stick to the sputum and cannot be washed away, while grains that have been mixed while

still moist with sputum are completely washed away. It is therefore clear that sputum from the lungs will be mixed with flat epithelial cells or some non-pathogenic microorganisms on its way to the mouth, but if the sputum is moist, the mixed elements will be completely washed away by the following method.

Washing.—A small specimen of the sputum is attached to the end of a platinum wire and immersed in a test-tube filled with isotonic salt solution. The material should be thoroughly rinsed with the platinum rod until it breaks up into several still smaller pieces. By washing the material once in this manner, it will usually be freed from contaminating bacteria, but sometimes the same technique must be repeated with fresh isotonic salt solution before it is cleared. The material is taken out of the salt solution, dried, and is then ready for examination.

Film Preparation. Fresh Material.—For the purpose of demonstrating the blood in the sputum fresh material is most suitable. The material should be washed as described above and placed on the slide. The cover-glass is then pressed lightly with the tip of the finger in order to make the film of the sputum suitable for microscopical examination.

Stained Material.—A small specimen of the washed sputum is put upon the middle of the slide and covered with a cover-glass, which is pressed lightly with the tip of the finger in order to obtain thin films. The cover-glass is then immediately pushed aside in contact with the surface of the slide, and removed, allowing the material to be dried in the air. If the material should be smeared with the platinum rod, the preparation is likely to be contaminated by the microorganisms attached to the rod.

Fixation.—For the demonstration of the blood in the sputum, the material is fixed by immersing it in absolute alcohol for 30 to 60 minutes, or in pure methyl alcohol for 15 minutes. For the examination for tubercle bacilli the material can be merely passed over the flame. Care should be taken not to let the material become too hot, as this destroys the staining properties of the pus cells and other elements.

Staining.—The blood is demonstrated by the same method that is employed in the preparation of the stained blood film. For the

examination of the bacilli, Ziehl's carbol-fuchsin is used, which is then decolorized with a mixture consisting of 100 cc. of absolute alcohol and two drops of highly concentrated hydrochloric acid. By this procedure, the erythrocytes lose the hemoglobin content and have the appearance of a blank vesicle. Löffler's methylene blue may be substituted by borax-methylene blue, which is prepared by mixing 1 gm. of methylene blue with 2 gm. of borax and 100 cc. of distilled water. The mixture is left standing for over 2 months and is then ready for use. It may be still further diluted to 10 per cent. It is superior to Löffler's preparation, for the cells as well as bacilli take deeper stains, while not only the original mixture, but also the dilution can be kept for a long time.

Diagnosis.—The most important data by which the source of the sputum can be determined are as follows: the erythrocytes or bacilli must be included in the same membrane of the mucus with other elements of the sputum such as the epithelial cells, contaminating microorganisms, or white corpuscles, which indicate the origin of the sputum itself. The diagnosis can be established only when these bodies are found closely packed in the same membrane of the mucus.

Pulmonary Sputum.—(Figs. 1 and 3.) Pulmonary sputum may sometimes contain round or elliptical epithelial cells a little larger than pus cells, but it never contains polygonal flat cells with nuclei. Theoretically, polygonal flat cells without nuclei may be found in the pulmonary sputum, but I have never observed any. Excepting the cases of mixed infection, one, or rarely two species of bacteria besides tubercle bacilli may be found. In no case does it contain an abundant number of different species of bacilli. If the clean sputum contains erythrocytes, the diagnosis of pulmonary hemorrhage may be made. If the same specimen should prove positive for tubercle bacilli by staining, the hemorrhage may be concluded to have occurred in the tuberculous lesions in the lungs.

Laryngeal Sputum.—(Figs. 2 and 4.) Laryngeal sputum usually contains polygonal flat cells with a large number of numerous species of microorganisms. It may contain only a few of these elements, but very rarely. If the laryngeal sputum contains erythrocytes the hemorrhage has occurred above the larynx. Again, if it is found to

contain tubercle bacilli, the diagnosis will be laryngeal or pharyngeal tuberculosis.

Nasal Secretions.—The nasal secretion of an acute nasal catarrh never contains flat epithelial cells or ciliated cylindrical epithelial cells, but may present the features of pulmonary sputum under the microscope. It usually contains, unlike the pulmonary sputum, various species of microorganisms, but sometimes contains only a few cocci or influenza bacilli. In such a case, it is difficult to establish a diagnosis by microscopical examination. In chronic nasal catarrh polygonal flat cells are always met with, but sometimes the material may contain only a few non-pathogenic contaminating bacilli. The secretion of the nasolaryngeal cavities always contains flat epithelial cells and various species of microorganisms, and presents the same features as the pharyngeal or laryngeal sputum.

By the method of examination described above, the source of the sputum can be determined and the diagnosis is thus facilitated. After several washings the material becomes free of the extraneous elements which may have come in contact with it; *e.g.*, polygonal epithelial cells. These polygonal flat epithelial cells under such circumstances will be found to be few in number, seldom exceeding two or three, and different in appearance from those of the laryngeal sputum. Moreover, in the pulmonary sputum these flat cells do not stick together with the tubercle bacilli or erythrocytes. By careful treatment and washing of the material, reliable results may be obtained. Saliva will be dissolved by the isotonic salt solution when it is washed, and completely separated from the materials together with any solid elements that might have been contained in it. If, again, two specimens of sputa are placed in contact with each other, they will not stick together unless they become dry. By washing a specimen of moist sputum, all the attached particles will be completely separated and removed from the material. By this means error in examination is avoided. If all the erythrocytes are removed by washing the material several times, the source of the hemorrhage is above the larynx.

I detected erythrocytes in the pulmonary sputum of all cases of hemoptysis examined immediately after pulmonary hemorrhage. The sputum from patients with laryngeal tuberculosis showed tubercle

bacilli in every instance. Laryngeal sputum is often so difficult to obtain that a number of examinations are usually required. Large masses of laryngeal tuberculosis sputum are seldom found; the specimens are usually small and mixed with saliva. A diagnosis, therefore, cannot always be established even after two or three examinations. From patients with both laryngeal and pulmonary tuberculosis two kinds of sputum are obtained and both must be examined. I have diagnosed the early stage of laryngeal tuberculosis in 6 out of 150 patients in whom no symptoms of the disease were evident.

Bacteriological examination of the laryngeal sputum of frank cases of laryngeal tuberculosis frequently showed tubercle bacilli; in some cases, however, scores of examinations were made before the contaminated sputum was obtained.

SUMMARY.

1. Microscopical examination of sputum that has been washed with isotonic salt solution indicates its source. Sputum from above the vocal cords contains polygonal flat epithelial cells and numerous species of non-pathogenic microorganisms. Sputum from below the cords is clear of saprophytes, although it sometimes contains bronchoalveolar cuboidal cells.

2. The source of the sputum can be determined by the erythrocytes and the bacilli that are contained in it, and the site of the lesion can also be ascertained.

3. In every instance I found erythrocytes in the pulmonary sputum after severe pulmonary hemorrhage.

4. Six of the cases of laryngeal affection referred to in the present paper had had no subjective symptoms, but microscopical examination showed tubercle bacilli in the laryngeal sputum. All the six cases were examined by Dr. Tanaka and were found to have tuberculous lesions in the larynx. In the laryngeal sputum of most cases of laryngeal tuberculosis with symptoms, tubercle bacilli have been frequently demonstrated, while in some cases contaminated laryngeal sputum was obtained only after a long series of examinations.

5. Microscopical examination of the sputum plays a significant part in the diagnosis of tuberculosis.

In conclusion, I wish to express my indebtedness to Dr. Tanaka, laryngologist, who supervised the laryngeal examinations for me, and to Messrs. Nemoto and Shiiba for their assistance.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. Sputum from a patient who had a pulmonary hemorrhage (about 30 cc.); fresh specimen. Leitz oc. 1, obj. $\frac{1}{12}$, oil immersion. The stained preparation from the same material shows tubercle bacilli corresponding to No. V of Gaffky's scale.

FIG. 2. Laryngeal sputum from a patient with laryngitis; fresh specimen. Leitz oc. 1, obj. 4. Numerous polygonal flat epithelial cells and a few white corpuscles are seen. The stained preparation of the same material shows also numerous saprophytes. No tubercle bacilli were seen.

PLATE 33.

FIG. 3. Pulmonary sputum. Stained preparation. Leitz oc. 1, obj. $\frac{1}{12}$, oil immersion. White corpuscles, tubercle bacilli, and a cuboidal cell are seen.

FIG. 4. Sputum from a patient with laryngeal tuberculosis. Stained preparation. Leitz oc. 1, obj. $\frac{1}{12}$, oil immersion. White corpuscles, most of which have been destroyed, and tubercle bacilli are seen with the polygonal flat epithelial cells and numerous saprophytes, especially cocci and bacilli.

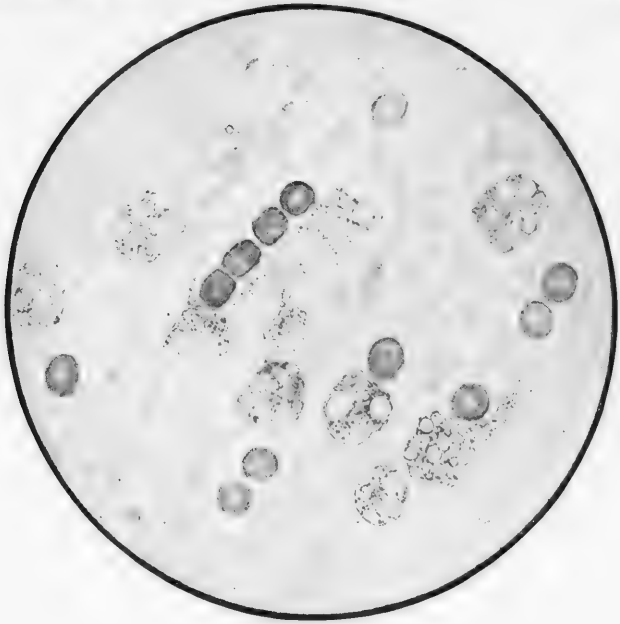

FIG. 1.

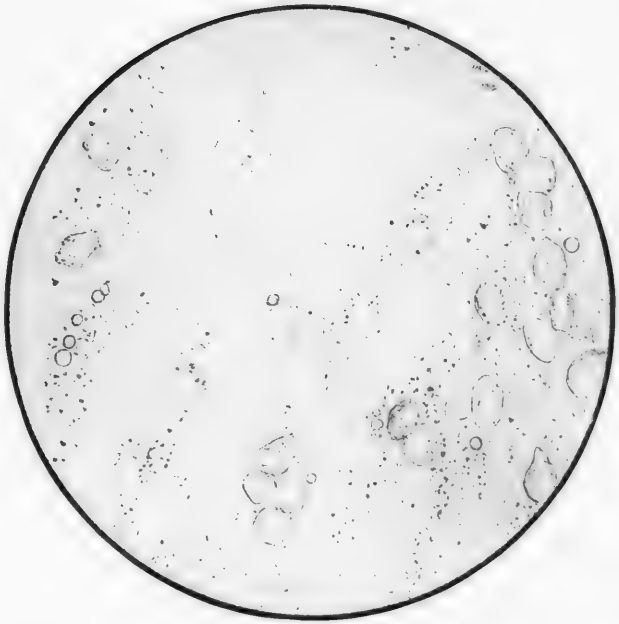

FIG. 2.

(Otani: Epithelial Cells and Saprophytes in Sputum.)

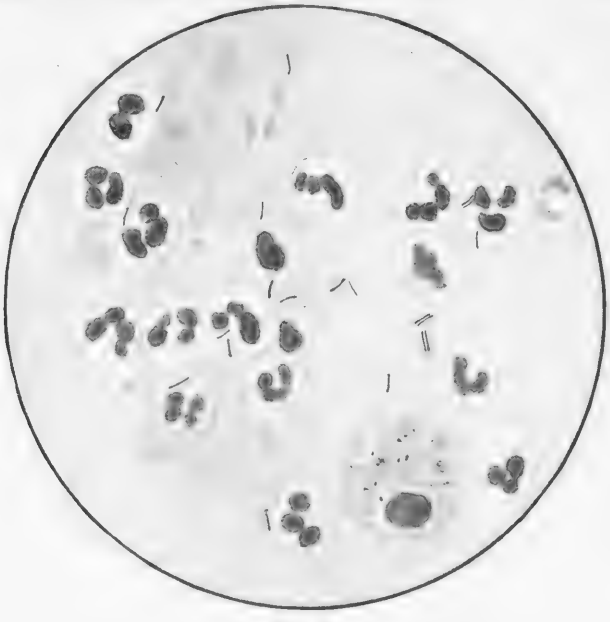

FIG. 3.

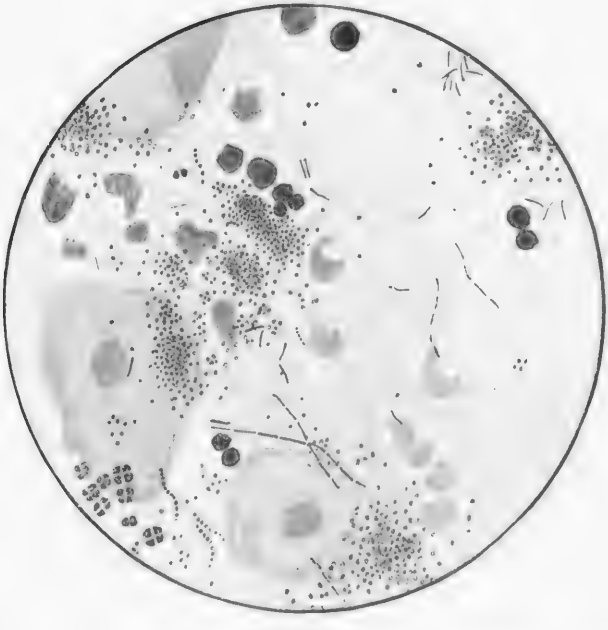

FIG. 4.

(Otani: Epithelial Cells and Saprophytes in Sputum.)

THE CULTIVATION OF TRICHOMONAS OF THE HUMAN MOUTH (TETRATRICHOMONAS HOMINIS).

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PLATES 34 TO 37.

(Received for publication, October 15, 1916.)

Few attempts have been made to cultivate the parasitic flagellates of man. The first report on the cultivation of the trichomonas was published by Lynch (1) in 1915.

Lynch found numerous trichomonades in the vagina and gums of a negress who was suffering from catarrhal vaginitis and acute gingivitis. He placed the material in neutral and acid bouillon and kept it alive for 3 days at 30°C. Then, as the organisms began to be affected by bacterial growth he transferred them to new media and was thus able to carry the culture through several generations.

The experiment described in this communication was carried out at first without the knowledge of Lynch's report and differs considerably from his.

Material.—The tartar of the mouth which has long been known to harbor trichomonades was used. In most instances they are few in number.[1]

Culture Media.—A number of different media were employed. Acid bouillon, neutral bouillon, Ringer's solution, ascitic fluid, ascitic bouillon (equal parts), ascitic fluid-isotonic salt solution (equal parts), ascitic fluid-Ringer's solution (equal parts), bouillon-Ringer's solution, sheep serum water, sheep serum water-Ringer's solution (equal parts), rabbit serum water, nutrient agar, ascitic agar, ascitic Ringer's agar, sheep serum-glucose agar. Fresh tissue was placed in both the fluid and solid media.

Most of the media were unsuitable; only a few gave relatively good results; the mixture of ascitic fluid and Ringer's solution in equal

[1] We wish to express here our indebtedness to Dr. M. I. Schamberg for his courtesy in providing us with some of the materials used for the present study.

portions and in its natural alkalinity gave entire satisfaction. Samples of ascitic fluid from different sources were used and gave equally good results. The bouillon employed by Lynch was not so suitable as our ascitic Ringer's solution, the chief reason probably being that it enables the contaminating bacteria to multiply so rapidly and profusely that the trichomonades no longer find it a suitable medium. Undiluted ascitic fluid was also found to be too rich in protein material, although in this respect it is more suitable than bouillon.

Culture.—When the culture medium is inoculated with a small amount of dental tartar, the trichomonades are so few that it takes some time to find one. But by placing the inoculated tubes at a temperature of 37°C.[2] and then transferring a small quantity of the culture into new medium every 24 hours for 4 or 5 days in succession, the number of the organisms gradually increases and no difficulty was experienced in finding quantities of agglomerating flagellates as well as free individuals in every field. The organisms group together in clusters of varying size around the masses of bacteria (Fig. 1). At lower temperatures (23–27°C.) the growth of bacteria as well as of protozoa proceeds much more slowly than at higher temperatures, and the transfer may be made every 48 hours.

The method of transfer is simple. As the trichomonas grows at the bottom of the tube with bacterial sediments, it is necessary to suck up this precipitate with a capillary pipette and transfer it to new tubes. In both sucking up and transferring precaution should be taken not to stir the culture too much. In this way strains were carried for over twenty generations, and the loss of the strain was usually due to an accident or negligence in the transfer.

A brief note on the *Entamœba gingivalis* Gross (*Entamœba buccalis* Steinberg), which is almost always encountered in this medium, may be made here. In this medium the entameba can easily be kept alive over 24 hours, and with a little precaution sometimes over 48 hours; that is, by keeping the medium always at about 37°C. and by having a sufficient amount of culture fluid. If the alkaline reaction of the medium is reduced by adding drops of a 1 per cent solution of acetic acid to almost the neutral point indicated by litmus paper,

[2] Lynch could not get cultures at 37°C.

the rapidity of the growth of the trichomonas is somewhat reduced, whereas that of the entamebæ is accelerated. By transferring the sediments into a new, already heated medium once every 24 hours, the amebæ can be kept alive over a week; in one instance they were kept alive as long as 10 days. Judging from the fact that only a small amount of the sediment is carried from one culture tube into two, three, or more new tubes each time, the amebæ remain alive and multiply, though not so strikingly as in the case of the trichomonades.

Morphological and Biological Properties of the Culture Trichomonas.

The body of the trichomonas is uniformly pear-shaped; but being highly flexible it can take any shape according to circumstances, especially at the time when it passes through the narrow spaces in the bacterial masses, as observed in a fresh preparation. The protrusion of any definite pseudopodium as drawn by Kuczynski (2) in the case of *Trichomonas augusta* has not been seen in our culture, but a prolongation of the protoplasm near the posterior end (Fig. 2) was often observed. This is often used as an anchor, while the main part has a lively oscillating movement (von Prowazek's *haftpseudopodialer Fortsatz*). This prolongation of the protoplasm, however, has nothing to do with a real pseudopodium as we understand it in the case of the ameba, and there is no evidence that it is used for obtaining food. On the other hand, the presence of a cytostome (peristome) is probable, though we could not discern it clearly. In one instance a long chain of bacilli was gradually taken in through a point near the beginning of the flagella. After it became embedded in the protoplasm the chain broke into two parts, one of which was soon cast off from the body through the same point by which it had first entered, while the other seemed to be digested.

The size of the culture trichomonas varies within wide ranges, but it usually measures 10 to 15 μ in length and 4 to 8 μ in width (Fig. 11). Sometimes particularly large forms are encountered, about 25 μ long and 12 μ wide (Figs. 12 and 13). Flagella at the anterior end are uniformly four in number, radiating out from a basal granule situated just in front of the nucleus. They are unequal in length, in most instances

two of them being a little longer than the other two. The length of the flagella averages 14 to 16 μ. An undulating membrane rising from a blepharoplast, which lies close to, but differs from the basal granules, is distinctly seen (Fig. 8). The distal end of the limiting line of the membrane usually lies within the body, while its terminal end sometimes projects beyond the margin as a short, free flagellum (Fig. 3).

The axostyle usually seems to take its origin at the distal end of the spindle-shaped nucleus, but sometimes its course may be traced through the nucleus as far up as the basal granule, or very close to it. In its distal course the axostyle runs downward through the body and projects beyond the margin at the posterior end of the organism, forming a free tail 5 μ or less in length. The nucleus is single, oval, more or less elongated in shape, has a distinct karyosome, and measures 1 to 1.5 μ by 2 to 3 μ.

The protoplasm shows no differentiation of ectoplasm and endoplasm, and no contractile vacuoles or chromidial apparatus could be observed other than fine granulation with scattered, coarse particles. In some specimens the protoplasm is filled with ingested bacteria. In preparations stained with Giemsa's solution two or more characteristic purple rings are found, not unlike the forms described by some authors as a stage of so called cyst formation (Fig. 6).

The common method of reproduction seems to be a binary, longitudinal fission (Figs. 14, 15, 16, 17, and 18); multiple fission (or rather budding) may also occur in the culture. Figs. 2 to 10 show a series of successive stages of division, which is complete within from 30 minutes to 1 hour in the hanging drop. The number of daughter cells of multiple fission is not great (4, 6, 8, etc.); they do not seem to separate from each other at the same time, but usually bud off one after another in succession, so that the general impression differs considerably from the usual mode of schizogony as in other protozoa (Figs. 19 and 20). The division of blepharoplast is of the mitotic type (Fig. 19), while that of the nucleus itself is promitotic (Fig. 21). In cultures no form has been observed which may be interpreted as a cyst.

DISCUSSION AND SUMMARY.

Trichomonades from the mouth were studied by Steinberg[3] who proposed to group them into three distinct types; namely, *Trichomonas elongata*, *Trichomonas caudata*, and *Trichomonas flagellata*. Doflein (3) regards them as probably identical with *Trichomonas hominis*. Opinions differ as to whether or not *Trichomonas vaginalis* Donné and *Trichomonas hominis* Grassi are the same species. Lynch, for instance, believes that they are the same species, while von Prowazek (4), Bensen (5), and others (6, 7) insist that they are different types. Bensen's view seems to be well supported by the difference alleged to be found between the mode of encystment in the two trichomonades, were it not for the fact that our knowledge about the so called cyst of trichomonades is still obscure. According to Alexeieff (8) many of the so called cysts were evidently blastomyces contained in the cell body of the trichomonas. An autogamy alleged to take place in cysts as described by Bohne and von Prowazek (9) has not been confirmed by Dobell (10). And Wenyon (11) contends that it has never been found possible to produce any development of these cysts outside the body on the warm stage as can be done with the cysts of *Entamœba coli*. Therefore, it is still premature to take the process of encystment into consideration as far as the classification of trichomonas is concerned. On the other hand, Rodenwaldt (12) seems to think that there are many species of trichomonas in the human intestines, and Wenyon has described a new trichomonas from the human intestines (*Macrostoma mesnili* Wenyon).

Further cultural studies in the morphology and biology of these organisms must be carried out in order to solve these problems.

In the light of modern investigations there are five subgenera to be included under the genus *Trichomonas* Donné. They are as follows:

(1) *Protrichomonas* Alexeieff, with three anterior flagella, without an undulating membrane.

(2) *Trichomastix* Bütschli, with three anterior flagella and a trailing flagellum (*Schleppgeissel*) without an undulating membrane.

[3] Steinberg, quoted by Doflein, F., in Lehrbuch der Protozoenkunde, Jena, 3rd edition, 1911, 492.

(3) *Trichomonas* Donné, with three anterior flagella and an undulating membrane.

(4) *Macrostoma* Alexeieff, Amend, Wenyon (11), with three anterior flagella and an undulating membrane wedged in a deep groove (peristome).

(5) *Tetratrichomonas* Parisi (13), with four anterior flagella and an undulating membrane.

As far as our culture trichomonas from the human mouth is concerned, it has been shown that it is not strictly a trichomonas and that it should be classed under the subgenus *Tetratrichomonas*.

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EXPLANATION OF PLATES.

All the specimens were obtained from cultures of *Tetratrichomonas hominis*.

PLATE 34.

FIG. 1. Dark-field illumination of a colony of *Tetratrichomonas hominis* in culture. × 1,000.

PLATE 35.

Air-dried, fixed in methyl alcohol, and stained with Giemsa's solution. Drawn with camera lucida, oc. 4, obj. $\frac{1}{12}$, oil immersion. Tube 160.

FIG. 2. Typical shape.

FIGS. 3 to 9. Different shapes.

FIG. 10. A phase of binary fission.

PLATE 36.

Moist, fixed in Schaudinn's sublimate alcohol with a few drops of acetic acid, and stained with Heidenhain's iron and hematoxylin.

FIG. 11. Small type.

FIGS. 12 and 13. Large types.

FIGS. 14 and 15. Preliminary phases of binary fission.

FIG. 16. Beginning of binary, longitudinal fission of the protoplasm.

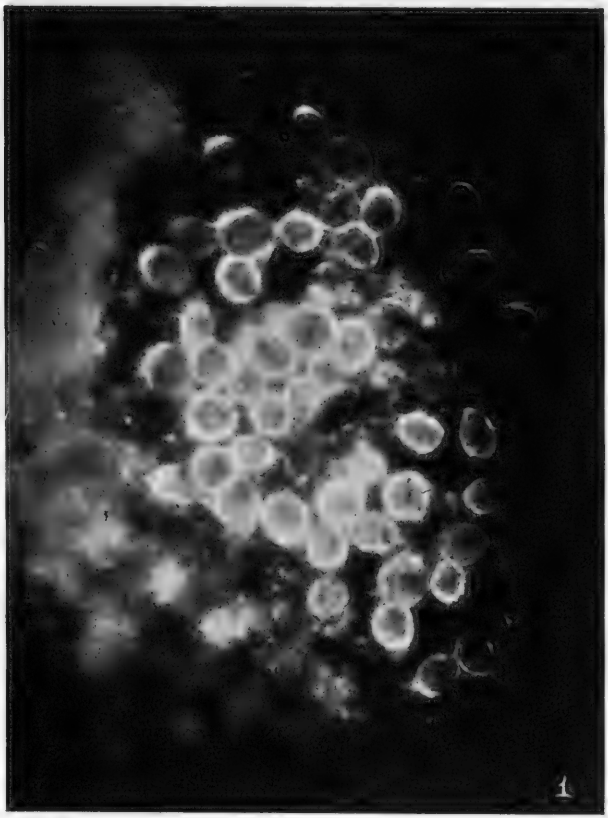
FIGS. 17 and 18. Final phases.

PLATE 37.

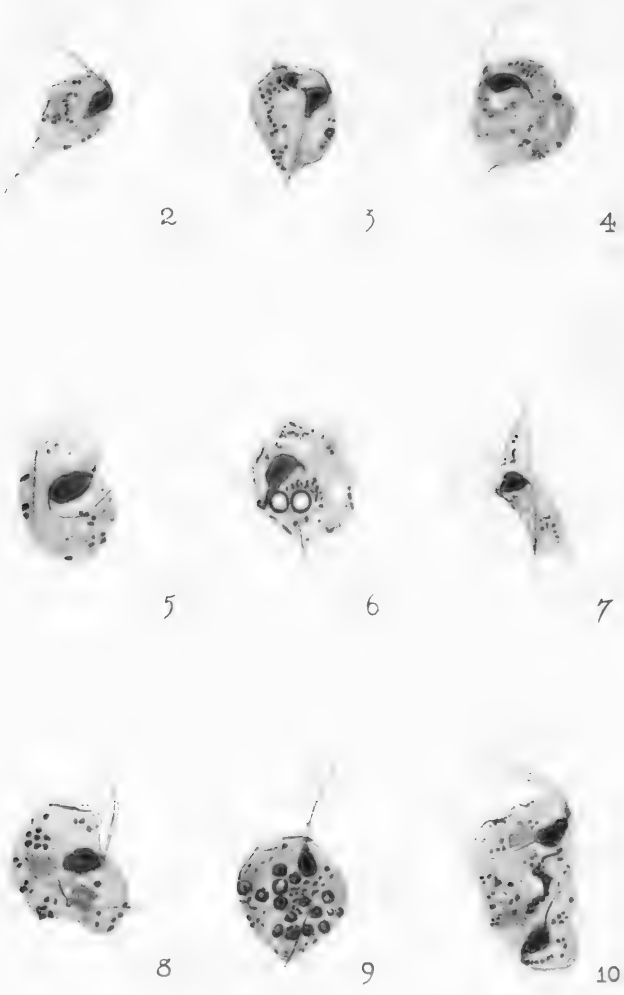
FIG. 19. A phase of multiple fission, showing six daughter nuclei and a mitotic spindle of a basal granule.

FIG. 20. Final stage.

FIG. 21. Promitotic division of the nucleus.



(Ohira and Noguchi: Trichomonas of the Human Mouth.)



Tokuzo Ohira fec. (Ohira and Noguchi: Trichomonas of the Human Mouth.)

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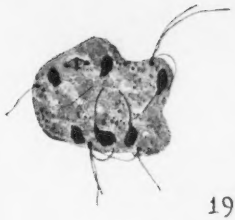
Tokuzo Ohira fec.

(Ohira and Noguchi: Trichomonas of the Human Mouth.)



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Tokuzo Ohira fec.

(Ohira and Noguchi: Trichomonas of the Human Mouth.)